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Effects of Ammonium and Phosphate Concentration on Growth, Pigment and Soluble Protein concentration in *Gracilaria manilaensis*

Alireza Joniyas*[1], Misni Surif[1], Norsuhana Abdul Hamid[2], Raheleh Dehgahi[3]

[1]*Centre for Marine and Coastal Research, Universiti of Sains Malaysia, Malaysia*

[2]*Biology program, School of Distance Education, Universiti of Sains Malaysia, Malaysia*

[3]*School of Biology, Universiti of Sains Malaysia, Malaysia*

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Abstract

Gracilaria is a genus of red algae (Rhodophyta) which is an important raw material for agar industry and some other industries. Nutrient is important environmental factors in seaweed growth. *Gracilaria* has high potential for absorbed of nutrient in sea water. The following work is devised on the basis of impact of variable concentrations of nitrogen and phosphorous which are 0, 20/2, 50/5, 120/12 and 300/30 µM. The factors determined to be affected were protein soluble, growth and pigments in red seaweed *Gracilaria manilaensis*. Results stated that concentration alteration of N and P affected the specific growth rate, total soluble proteins and chemical composition (concentrations of chlorophyll a, phycoerythrin (PE), phycocyanin (PC)). N and P concentration was increased, the growth rate of *Gracilaria manilaensis* were increased 2.97± 0.26%d^{-1}, in 0 concentration of ammonium to 5.72± 0.19%d^{-1} in 300/30 µM . One -way ANOVA showed significant differences in growth rate between treatments and cultivation time ($P<0.05$). Growth rates were 50% higher in 300/30 N/P concentration compared to without nutrient treatments. Similarly, PE, PC and Chl a were increased and highest value were found at N/P concentration of 300/30 µM. Growth rate of *G. manilaensis* under different concentrations of N and P was strongly dependent on the concentrations of inorganic concentration N and P.

* **Corresponding Author:** Alireza Joniyas ✉ a_jon58@yahoo.com

Introduction

Enhanced growth of mariculture affects the environment adversely. In mariculture, the high N/P concentrations are hazardous for the environment (Feng *et al.*, 2004). The studies are widely conducted in order to improve the quality of water and decrease the accumulation of N/P in mariculture. Consequently, co-culture of seaweeds is thought to be the solution of this problem. In co-culture, nutrients are used efficiently and eutrophication is prevented. Co-culture also resulted to prevention of red tides and diseases (Yang *et al.*, 2006; Troell *et al.*, 2006).

Nutrient is the main factor for controlling the physiological functions in seaweeds. Nitrogen is important as it restricts the growth in temperate waters (Abreu *et al.*, 2011). Algal growth requires nitrate and phosphate significantly; absence of any one restricts the growth (Lapointe, 1987). Conversely, in natural habitat, the plasmalemma exhibits the active transportation of nutrients as nutrient concentrations could be 1000-fold in cell environment compare to cell inside. Uptake of nutrients is dependent on physical, biological and chemical factors. Physical contains light, water motion, temperature and desiccation (Lobban and Harrison, 1994) Ammonium uptake is affected by irradiance whereas, uptake of nitrate and phosphate is affected by spectral quality (Pedersen *et al.*, 2004b).

Photosynthetic products provide the energy for nitrogen fixation process in plant and ammonium is the main component of nitrogen fixation. Moreover, ammonium is lethal for plants; therefore, it is quickly converted into amino acids (Vinod and Heuer, 2012). Some activity in plants require nutrients to live in shallow coastal waters (Lobban and Harrison, 1994).

Great number of plant species living in shallow water display different growth patterns and life forms. Growth of plants living under water is controlled by different factors including salinity, light and nutrients (Sand-Jensen and Borum, 1991; Duarte, 1995).

Macro algae will be distributed, if nutrition provided, while, limited nutrients resulted to slow growth (Kautsky *et al.*, 1986; Ménesguen and Piriou, 1995; Taylor *et al.*, 1995; Borum and Sand-Jensen, 1996).

As a first phase to explain the bio-remediation capability of *Gracilaria manilaensis*, the present in this experiment quantified growth rate and removal of nitrogen over the range different level of ammonium. An supplementary answer variable were chlorophyll a, phycocyanin and phycoerythrin, a proteinaceous pigment unique to *Gracilaria* family (Harrison and Hurd, 2001). Representing as much as 85-87% of total phycobiliprotein, this pigment accumulates quickly at high nitrogen concentrations, subsequent in improved photosynthetic activity and growth (Carmona *et al.* 2006). In the context of growth, bioremediation, it was important to include pigment content at last total soluble protein content measurement here.

The objective of the present study was to carry out and determine the effect of different concentrations of phosphorous and ammonium on growth rate, chlorophyll a, PE (phycoerythrin), PC (phycocyanin) and total soluble protein of red seaweed *G. manilaensis*.

Materials and methods

Study area and sample preparation

Gracilaria manilaensis was gathered from a culture pond present in Kota Kuala Muda, Kampong Bukit Berangan, and Kedah, Malaysia, The specie was placed in a polystyrene box which contained ice in order to keep the plant chilled. The plant was taken to the laboratory and epiphytes and dirt was removed from it (Dawes and Koch, 1991).

The sample plant was kept in an aquarium comprising salinity of 25ppt which was artificial seawater. The plant was adjusted in the following conditions for 10 days. Conditions included aeration and light intensity 50 μmol photons m^{-2} s^{-1}(flowed from (Kumer *et al.*,2010), (SUNS INTERNATINAL LCC, model: LX-101, USA).

The plant was exposed to these conditions for 12h in day and 12h in night for providing dark hence, photoperiod becomes 12hL: 12hD (flowd fram Yu and Yang (2008)). Later, when the plant is adjusted, 3g of *G. manilaensis* were transferred to Erlenmeyer flasks (1L) which possessed 500 ml artificial seawater (ASW, Instant Ocean by Aquarium Systems, France).

Enrichment was performed in the artificial seawater by adding 50µM NH_4^+ 5µM PO_4. Five N/P were used which were 0, 20/2, 50/5, 120/12 and 300/30 and each was run in triple replicates. The experiment was carried out for 10 days ((Kumer *et al.*,2010).) unless the pigment, soluble proteins and growth (SGR) were estimated.

Experimental data determination

Growth rate

The specific growth rate (SGR) was used to evaluate growth according to an illustration by (Campbell, 2001). The specific growth rate was calculated as: SGR, µ (%d^{-1}) = ($\ln B_t - \ln B_0$) t^{-1} x 100 Where, where B_0 = initial weight of sample Bt = final weight of sample t = day.

Pigment

Macroalgal chlorophyll content was extracted from fresh tissues (0.6g) *G. manilaensis* with

10 mL of 80% acetone kept in darkness overnight at 4 °C and determined according (Dawes *et al.*, 1998). At 665nm, the absorbance was recorded through Hatch DR 2800 spectrophotometer (USA).

Extraction of PE and PC was conducted by 0.1 M phosphate buffer (pH=6.8). The concentrations of these PE and PC were estimated by absorbance using Hatch DR 800 spectrophotometer at wavelength of 455nm, 564nm, and 592nm for PC and 645nm and 618nm for PE (Beer and Eshel, 1985).

Determination and Protein Assay (TSP)

The total soluble protein contents of the *G. manilaensis* under salt stress were determined by using the method of (Bradford, 1976).

The total soluble protein assay was carried out by addition of 20 µl of sample extract to 80 µl of protein extraction buffer and 5 ml of protein reagent.

The solution was thoroughly mixed by vortexing, followed by a determination of absorbance at 595 nm using (Hitachi U1900, USA) spectrophotometer. The total soluble protein content of the samples was determined from the standard curve plotted by using bovine serum albumin (BSA; Sigma Aldrich, USA) as the standard at 20, 40, 60, 80 and 100 µg/mL The total soluble protein content of the samples was then expressed in mg/g FW of the plant material.

Statistical analysis

To analyse the data, one way analysis of variance (ANOVA) was used. In this regard, Tukey HD was used as the post hoc test to compare between group significance. All the analysis was performed using Statistical Package for Social Sciences (SPSS) software version 22 (Dehgahi *et al.*, 2015).

Results

Effect of Ammonium and Phosphate (N/P) Concentrations on the Growth of Gracilaria manilaensis

Result of the study showed that variations in ammonium concentration affect the growth of *G. manilaensis* (Fig,1). Results in (Fig2) howed SGR increased as N/P concentration increased from 0 µM to 300/30 µM. The lowest SGRs were observed at 0 µM N/P (2.97 ± 0.26% day^{-1}) and at 20/2, 50/5 120/12, and 300/30 µM ammonium to phosphate concentration. However, SGR increased to 3.53 ± 0.38, 4.41 ± 0.30, 5.30 ± 0.27, and 5.72 ± 0.19% day^{-1}, respectively (Table 1; Fig, 1).

Furthermore, the results indicated that significant differences were observed in SGR values of untreated *G. manilaensis* and those treated with 0, 20/2, 50/5, and both 120/12, and 300/30 µM ammonium : phosphate, although no statistically significant differences were observed between the two concentrations of 120/12 µM and 300/30 µM (N/P) and SGR.

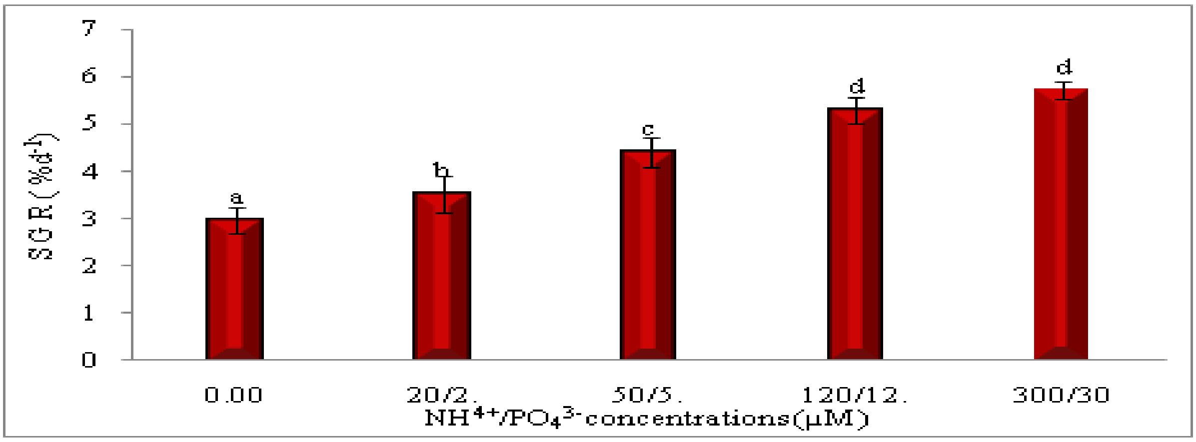

Fig. 1. Efect of different N/P concentrations on the Specific Growth Rate (SGR) of *Gracilaria manilaensis*. Results are shown as mean ± standard error (p < .05); (*n* = 3).

Effect of Ammonium and Phosphate (N/P) Concentration on Chlorophyll a Content in Gracilaria manilaensis

Chlorophyll a content of different N/P concentration treated and untreated *G. manilaensis* after 10 days of treatment revealed that a gradual increase in the chlorophyll a content was observed with increasing concentrations of N/P compared with initial values of *G. manilaensis* (Fig, 2).

The results from Tukey's HSD (p<0.05) indicated that all the chlorophyll a content of treated samples were significantly different compared with the initial concentration (4.22 ± 0.09 mg/g DW). Chlorophyll a content of untreated (0 concentration of N/P) *G. manilaensis* exhibited a reduction in chlorophyll a content (4.04 ± 0.17 mg/g DW), and was not significantly different from their initial content (Fig, 2).

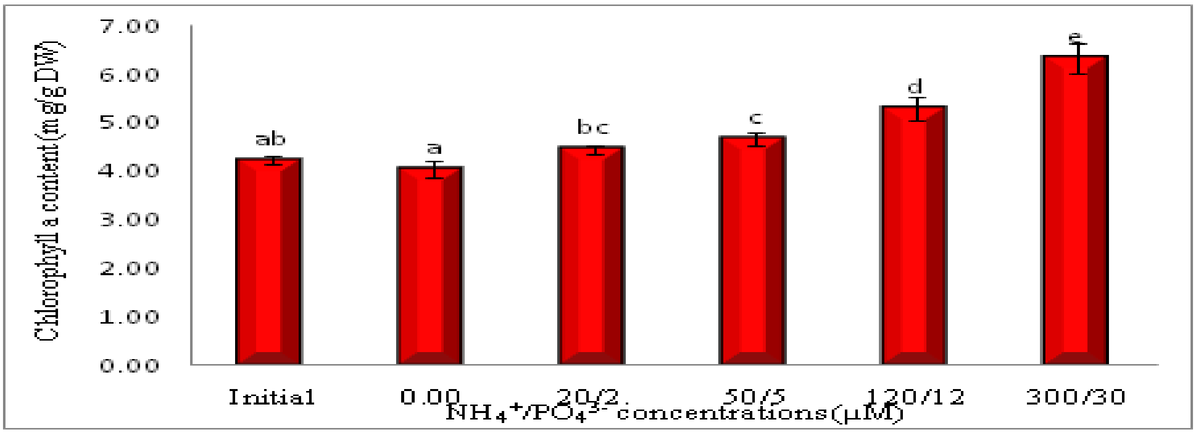

Fig. 2. Effect of different NH_4^+/PO_4^{3-} concentrations (N/P) on chlorophyll a content (mg/g DW) of *Gracilaria manilaens*. Results are shown as mean ± standard error (p < .05); (*n* = 3).

Furthermore, the results indicated that a significant difference was observed in chlorophyll a content of untreated *G. manilaensis* with those treated with all phosphate and ammonium concentrations.

Effect of Ammonium and Phosphate (N/P) Concentrations on the Phycoerythrine (PE) in Gracilaria manilaensis

The results of the study revealed that increases in N/P concentration have caused an increase in PE content (Fig, 3).

Phycoerythrine content in *G. manilaensis* cultured at 0 µM was 2.70 ± 0.18 mg/g DW, which is 4% less than the initial content. The PE content of *G. manilaensis* treated with N/P 20/2, 50/5, 120/12, and 300/30 µM concentrations were 3.06 ± 0.24, 3.07 ± 0.16, 3.40 ± 0.13, and 4.32 ± 0.30 mg/g DW, which are 9%, 10%, 21% and 54% greater than their respective initial PE content. The present study showed that a considerable increase of PE were seen in *G. manilaensis* treated at higher concentrations (300/30 µM) N/P. The present study showed that a considerable increase was seen in *G. manilaensis* treated at higher concentrations (120/12 and 300/30 µM of N/P). Furthermore, they were not significantly different from one another and lower concentration in treated samples except under 300/30 µM N/P (Tukey's HSD test p 0< .05).

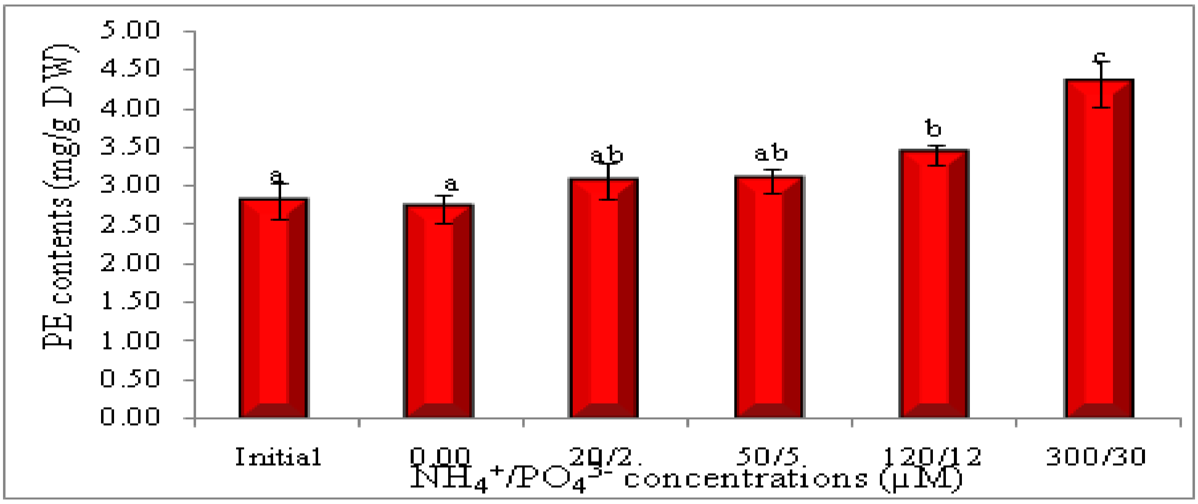

Fig. 3. Effect of different N/P concentrations in Phycoerythrine=PE content (mg/g DW) of *Gracilaria manilaensis*. Results are shown as mean± standard error (p<0.05); (n=3).

Effect of Ammonium and Phosphate (N/P) Concentrations on the Phycocyanine (PC) Content in Gracilaria manilaensis

The results of the study revealed that increases in N/P concentrations caused increases in PC content (Fig, 4). The present study showed that various concentrations of N/P have caused different PC contents compared with their respective initial concentration of PC in *G. manilaensis* (1.27 ± 0.18 mg/g DW).

An increasing trend in PC contents was observed at the end of the treatment in *G.manilaensis* treated with various N/P concentrations (Figure 5). At the end of the 10 days experimental period, *G. manilaensis* samples subjected to 0 µM of N/P displayed less PC content (1.22 ± 0.09 mg/g DW) and were not significantly different (p < .05) from the initial samples (Table 1).

Gracilaria manilaensis treated with 20/2, 50/5, 120/12, and 300/30 µM of N/P exhibited PC contents of 1.34 ± 0.20, 1.40 ± 0.18, 1.61 ± 0.13 and 2.20 ± 0.10 mg/g DW, respectively. The results indicated that PC contents of *G. manilaensis* treated at 50/5, 120/12, and 300/30 µM of N/P were significantly different from the initial PC content (Tukey's HSD test p <0.05). PC contents of *G. manilaensis* treated with 20/2, 50/5, 120/12, and 300/30 µM of N/P were 5.20%, 10.23%, 26.70% and 73.2% higher than in their respective initial *G. Manilaensis*.

Effect of Ammonium and Phosphate (N/P) Concentration on Total Soluble Protein (TSP) Content in Gracilaria manilaensis

In this study, significant differences were found between the means of TSP content of *G. manilaensis* between different concentrations of N/P treatments (Table 1; Fig, 5). Results obtained revealed that TSP content increases as the concentrations of N/P increase.

In samples treated with 0, 20/2, 50/5, 120/12, and 300/30 µM of N/P, the TSP content in *G. manilaensis* were 8.68 ± 0.80, 11.69 ± 1.02, 14.34 ± 0.68, 15.38 ± 0.49, and 16.57 ± 0.91 mg/g DW respectively (Fig, 5). The TSP content of *G. manilaensis* cultured in 0 µM N/P concentration was 8.68 ± 0.80 mg/g DW, which is 5.7% less than the initial content, and the TSP content at N/P treatment 20/2, 50/5, 120/12, and 300/30 µM were increased by 26.9%, 55.7%, 67.0%, and 80.9% at the end of the experiment compared with their initial content. The differences between TSP content at different concentrations of N/P were statistically significant as per the Tukey's HSD ($p < 0.05$), except concentration 120/12 N/P compared with 50/5 and 300/30 µM N/P also, and no significant difference were found at concentrations 0 µ M N/P and the initial samples ($P < 0.05$).

Discussion

Seaweed has physiological mechanism to acquire, utilize and store various forms of nitrogen from the media. Successful cultivation of the seaweed requires a deep knowledge of the effect of nitrogen on seaweed growth. Nitrogen plays important roles in the photosynthesis, amount changes that have direct effect on the photosynthesis and growth.

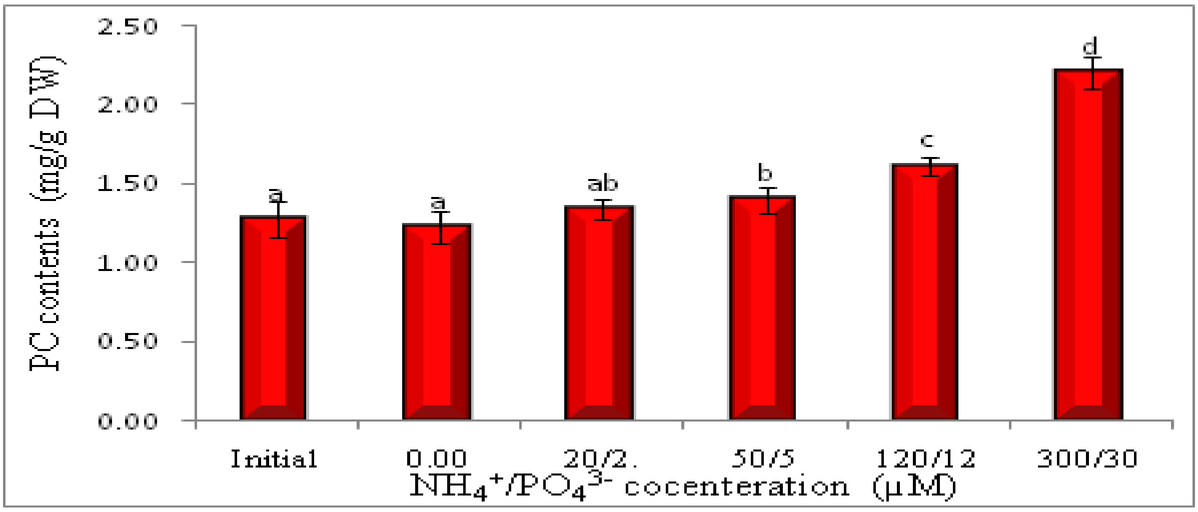

Fig. 4. Effect of different N/P concentrations in Phycocyanine = PC content (mg/g DW) of *Gracilaria manilaensis*. Results are shown as mean± standard error ($p<0.05$); (n=3).

Growth rate analyses demonstrated that N/P treatment at various concentrations have a significant effect on specific growth rate of *G. manilaensis*. The results of the present study indicated that the growth rate of *G. manilaensis* increases with the increase of N/P concentration, which may be due to higher supply of N/P. This result is in agreement with the finding of Hanisak (1990).

Similarly, a study by Korbee *et al.* (2005) on the relative growth rate of *Porphyra leucosticte* and *Porphyra umbilicalis* under different concentrations of ammonium found that ammonium fertility resulted in increase of the relative growth rates in both species. Yu and Yang, (2008) reported that specific growth rate of *G. lemaneiformis* under different N/P concentrations increased gradually with increasing nitrogen and phosphorus levels. However, they stated that at the highest N/P concentration (600/37.5 µM), specific growth rate of *G. lemaneiformis* decreased noticeably after 3, 7, and 15 days. Similarly, Israel *et al.*, (1999) pointed out that increase in $NH4^+$ concentration resulted in increased relative growth rate in *G. tenuistipitata* var. *liui*.

Ribeiro *et al.* (2013) investigated the relative growth rate of *Hypnea cervicornis* J. Agardh under different concentrations of ammonium and phosphate, in the range from 0 to 50 µM (10:1 nitrogen/phosphorus (N/P)).

They found that ammonium fertility increased the relative growth rate of the species. Growth rates of *H. cervicornis* increased linearly with the addition of ammonium and the highest growth rate was 11.5%d^{-1}.

Gorey *et al.* (2013) investigated the effect of ammonium and nitrate on growth rates of two species *P. palmata* and *C. crispus,* under five combinations of ammonium and nitrate, each with N concentration of 300 µM (300:0, 270:30, 150:150, 30:270, 0:300). Molar nitrogen/phosphorus ratio was 10:1. They reported that the maximum growth rates under 300 µM ammonium were 8.9% and 6.0% per day for *P. palmata* and *C. crispus*, respectively. Additionally, Lapointe and Ryther, (1978) reported that NH_4^+ and NO_3^- improve the growth of *G. tikvahiae* significantly. Literature shows *Gracilaria* species are adaptable to different concentrations of nitrogen for growth (De Boer *et al.*, 1979).

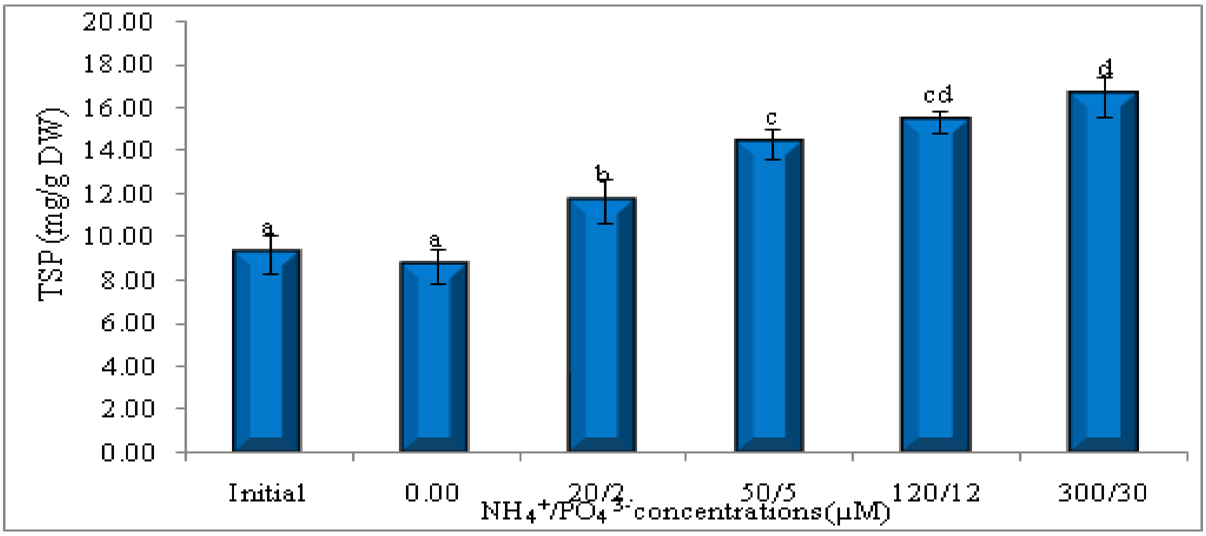

Fig. 5. Effect of different ammonium and phosphate (N/P) concentrations on total soluble protein (TSP) content (mg/g DW) in *Gracilaria manilaensis*. Results are shown as mean± standard error ($p < 0.05$); ($n = 3$).

In the process of photosynthesis in red seaweeds, pigments including chlorophyll a and PE play important role in converting light energy to chemical energy. The photosynthesis in algae is a cellular process. Korbee *et al.* (2005) reported that increasing ammonium concentrations to 300 µM resulted in increase of chlorophyll contents in *Porphyra leucosticte* and *Porphyra umbilicalis.*

Ribeiro *et al.* (2013) found that the highest content of chlorophyll a, PE and PC in *Hypnea cervicornis* J. Agardh were observed in the treatments of 30 to 50 µM ammonium at N/P ratio 10:1. Boderskov *et al.* (2015) investigated effect of nutrient on seasonal growth on *Saccharina latissimi.* They found that chlorophyll a content was less under low nutrient compared with under high nutrient levels. Yu and Yang, (2008) reported that increasing N/P concentration will increase chlorophyll a contents of *G. lemaneiformis* and concluded that increasing pigment content will accelerate photosynthesis and consequently increasing the growth of *G. lemaneiformis*. Other findings reported that higher concentrations of nitrogen and phosphors disrupt regular metabolism of photosynthesis and protein synthesis (Wilson and Gritchley, 1998; Peng *et al.*, 2007). Those findings are in agreement with that of Yu and Yang, (2008) who found that if the concentration of N/P is too high (600/37.5 µM), chlorophyll a contents will be reduced.

The results of this study showed that increase in N/P concentrations resulted to increase PE values in *G. manilaensis.* Similarly, Korbee *et al.* (2005) investigated the effect of different ammonium concentrations on PE content in *Porphyra umbilicalis* and *Porphyra leucosticta* for a duration of 3 days and 7 days.

Their results showed that 0 µM ammonium halted the production of PE. They also reported that increasing the ammonium concentration will increase PE content. Similar observation has also been reported by Yu and Yang, (2008) who highlighted that increasing the concentration of N/P will increase PE content in *G. lemaneiformis*. However, they found that if the concentration of N/P is too high, (600/37.5 µM) the production of PE will be inhibited, thus reducing the PE content.

This study also shows that increase in N/P concentrations will increase PC content in *G. manilaensis*. This finding is in agreement with that of the study by Korbee *et al*., (2005) who found that increasing the ammonium concentrations will increase PC content. However, their results showed that ammonium treated *Porphyra umbilicalis* and *Porphyra leucosticte* showed less PC content compared with the contents in the initial samples.
This study also reveals that increasing N/P concentration will increase in total soluble proteins (TSP). Some sea weeds such as *Porphyra* and *Ulva* contained 20% of their dry weight as protein (Fujiwara-Arasaki *et al*., 1984). Zhang *et al*. (2010) examined the effect of different N/P nutrient levels on soluble protein in *Potamogeton crispus*. They found that soluble protein content increases when seaweeds are fertilized with NH_4^+-N compared with untreated seaweeds.

Conclusion

In conclusion our proteomic study of the differential pigments and protein values under different nutrient concentration conditions found that *G. manilaensis* showed an increase trend as nutrient concentration. The effect of different nutrient concentration are probably responsible for the increase the level of pigments, protein and SGR.

Acknowledgement

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