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The suitable stocking density to growth of macroalgae *Spirogyra spp*. and its ability of ammonia nitrogen removal

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Studying the suitable stocking density to growth of *Spirogyra spp.* consisted of 5 treatments and 3 replications, *Spirogyra spp.* was reared in different stocking densities as 0 (control) 1 3 5 and 7 g fw (fresh weight) L^{-1} for 3 weeks and then studied its ability of ammonia nitrogen removal at the suitable stocking density for 3 days. The results indicated that weight gain and specific growth rate of *Spirogyra spp.* at all treatments were the highest in the first week of experiment and gradually decreased in the second and the third week respectively which weight gain and specific growth rate of *Spirogyra spp.* were cultivated at 1 g fw L^{-1} was higher than control treatment and other treatments. These parameters would decrease when stocking density increase. At the end of experiment, average weight gain and average specific growth rate of *Spirogyra spp.* rearing at 1 g fw L^{-1} were higher than other treatments (1.41±3.62 g and 5.57±4.56 %d^{-1} respectively) and showed significant difference ($p<0.05$) when compared with the treatments of 5 and 7 g fw L^{-1} but no significant difference ($p>0.05$) when compared with 3 g fw L^{-1} treatment. Finally, the suitable stocking density to growth of *Spirogyra spp.* in this study was 1 g fw L^{-1} which showed the best of weight gain and specific growth rate and its ability of ammonia nitrogen removal was 0.045±0.003 mg-N L^{-1} g fw^{-1} d^{-1}. In addition, *Spirogyra spp.* cultivation for 1 week revealed higher product than 2 and 3 week cultivation period.

Keywords: stocking density, *Spirogyra*, growth, ammonia nitrogen

Introduction

The genus *Spirogyra* has many potential for industrial applications which draw attention of researchers to study, mostly based on bioethanol and biodiesel potential (Eshaq *et al*., 2010; Adrien, 2011; Ramaraj *et al*., 2015). The biosorption potential of *Spirogyra sp.* can remove Cr 98.23%, Cu 89.6%, Fe 99.73%, Mn 99.6% Se 98.16% and Zn 81.53% after seven days of incubation

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period at 5 mg/l of initial metal concentration (Mane and Bhosle, 2012). Moreover, this valuable macroalgae have potential sources of proteases and can add to the pool of microbial sources of proteases for industrial applications and other biotechnological processes (Atawodi *et al*., 2015). With their potential in antioxidant and antibacterial activities, the methanolic and water extract from them can inhibit against *Escherichia coli* and *Candida albicans*. Besides, the principal potential of *Spirogyra* has been considered as food supplements that better than strains for bioethanol and biodiesel production (Adrien, 2011). Nutritional values of *Spirogyra* consist of 20.83% to 24.4% protein, 39.84% to 62.0% carbohydrate, 2.18% to 21.0% lipid, 10.66% moisture, 20.19% ash, 6.30% fibre and 117.42 to 387.22 mg of Ca per 100g of alga (Adrien, 2011; Tipnee *et al*., 2015).

The edible freshwater macroalgae, *Spirogyra*, is unbranced filmentous green algae of the family Zygnemataceae. Each cell has cylindrical shape with outer mucilaginous sheath and the chloroplast twists in from of apiral bands or ribbon-like. There are 22 species of *Spirogyra* existing in Thailand, the Thai common name is "Tao" or "Thao" or "Thao Nam" (Peerapornpisal, 2013). In natural water, they appear in zone of slow running water along the rivers or streams that form to be free-floating mats in shallow water. *Spirogyra* prefers to grow in the clean to moderate water quality, soluble ammonia nitrogen ranged form 0.32 to 0.75 mg/l, clear water with not exceeding 10 NTU of turbidity, 15 to 30.5°C of water temperature and pH 6 to 7.8 (Peerapornpisal, 2008; Thiamdao and Peerapornpisal, 2009; Sirirustananun and Chanartaeparporn, 2015). It can grow both resting on the sediment where has clay bottom and attached to substrates (Punyoyai, 2008; Sirirustananun and Chanartaeparporn, 2015). In northern of Thailand, These algae occur in cool-dry and summer seasons (Thiamdao and Peerapornpisal, 2011) corresponding with Phetchabun province, locate in the lower north of Thailand, Tao was found in wintertime from November untill Frebuary and presented again in June which has the end of summer and beginning of rainy season (Sirirustananun and Chanartaeparporn, 2015).

Currently, the popularity of Tao is continuously gaining as an ingredient or supplement for cosmetics, antioxidants in food as well as in pharmaceutical products (Lee, 1990) which isn't only interested in local areas but expanded to industrial units. Because of their qualities have high in nutritional values especially protein content comparable to fresh water fish, many vitamin and mineral particularly vitamin B and good tasty. For this reason, the local peoples use them as a source of food since the ancient times (Peerapornpisal, 2008). In the northern and north-eastern of Thailand, Tao was used as food called "Yum Tao" (Peerapornpisal, 2008; Peerapornpisal, 2013). In Phetchabun province, there is widespread consumption of Tao mainly of local peoples which collect productions from natural water and trade them in the local market. Tao's productions will find in some season according to the appearance in natural water. Therefore, Tao's productions from natural water are not enough to consumers requirement, the extension and development of Tao cultivation has the good practice to raising Tao's products in order to consumers satisfaction.

Light is an essential key for growth of algae by photosynthetic process which the optimal light exposure duration and frequency are important factors to cultivaion of more algae and at the same time optimize the land use (Ren, 2014). Algae rearing in the suitable stocking density can help to reduce light obstruction and competition of nutrients absorbtion particulary dissolved ammonia nitrogen that is a crucial nutrient for growth. The appearance of Tao in natural water will correspond with concentration of ammonia nitrogen rising (Sirirustananun and Chanartaeparporn, 2015) and its growth can help reducing the ammonia nitrogen in the water. Consequently, this study aims to determine the suitable stocking density to growth of *Spirogyra spp.* and its ability of ammonia nitrogen removal that much benefit to utilize for further development of Tao cultivation technic as well as water quality treatment technic.

Materials and methods

Experimental site and algae sample collection

The experiments were carried out at the aquaculture unit of agriculture technology faculty, Phetchabun Rajabhat University, Thailand. The freshwater macroalgae, *Spirogyra spp.* were collected from slow running freshwater stream at Ban Buchanuan, Nam Ron district, Amphoe Mueang Phetchabun, Thailand in February 2015. The collected algae were rinsed with stream water and submerged in stream water at 10 liters plastic box as container for transferred to experimental site.

Expermental 1: stocking density

The completely ramdomized design (CRD) was planned by dividing into five treatments and three replications. Five stocking densities of 0 (control treatment), 1, 3, 5 and 7 g fresh weight L^{-1} were tested. The algae were grown at different stocking densities for three weeks in 5 L clear plastic jar with the controlled total ammonia nitrogen water ranged from 0.75 to 1 mg-N L^{-1}. The culture medium had NH_4Cl as the source of nitrogen. The algae were weighed at the initial and the end of experiment as fresh weight (fw). During trial, once a week the biomass was recorded continually and the density was reduced back to the initial stocking density, applied from Pereira *et al.* (2006). Twice a week, the light density, the water temperature, pH, DO, and the total ammonia nitrogen of water samples were determined. The specific growth rates (SGR) were calculated using the following formula:

$$SGR\ (\%d^{-1}) = \frac{Ln\ (Wf) - Ln\ (Wi)}{T}\ 100$$

where: *Wf* = final wet weight; *Wi* = initial wet weight; *T* = days (Macchiavello and Bulboa, 2014)

Experiment 2: Ammonia nitrogen removal

Based on the results of the experiment described above (experiment 1), the suitable stocking density was used to cultivate algae with the controlled total ammonia nitrogen water ranged from 0.75 to 1 mg-N L^{-1} for three days. The algae were weighed as fresh weight (fw) and the total ammonia nitrogen (mg-N L^{-1}) of water samples were estimated at the initial and the end of experiment. The ability of total ammonia nitrogen removal was calculated as:

$$Nitrogen\ absorption\ rate\ (mg - N\ L^{-1} g\ fw^{-1} d^{-1}) = \frac{Nf - Ni}{B \times t} \times \frac{1}{\bar{N}}$$

where: *Nf*= nutrient content in water at the end of the experiment; *Ni* = nutrient content in water at the beginning of the experiment; $\bar{N}$ = *(Nf* + *Ni)*/2; *B* = initial biomass; *t* = days (Feijoó *et al.*, 2002)

Statistical analysis

For all treatments, the three replicates were analyzed means and standard deviations. Differences among treatments were tested for significance using one-way ANOVA. Multiple posthoc comparisons among means were tested by the Duncan's multiple range test (DMRT) at 0.05 of significance level. All analyses were performed with IBM SPSS statistics version 21.

Results

The cultivation of *Spirogyra spp.* in different stocking densities as 0 (control treatment), 1, 3, 5 and 7 g fw L^{-1} for 3 weeks, the results indicated that weight gain and specific growth rate of *Spirogyra spp.* at all treatments were the highest in the first week of experiment and gradually decreased in the second and the third week respectively. In each week, weight gain and specific growth rate of algae were reared at 1 g fw L^{-1} was higher than other treatments as the lowest of stocking density level in this study. For algae were reared at 5 and 7 g fw L^{-1} showed no weight gain and negative of specific growth rate since the first week of experiment. Weight gain and specific growth rate of algae trended to be decreasing when stocking densities increased (Fig. 1 and 2).

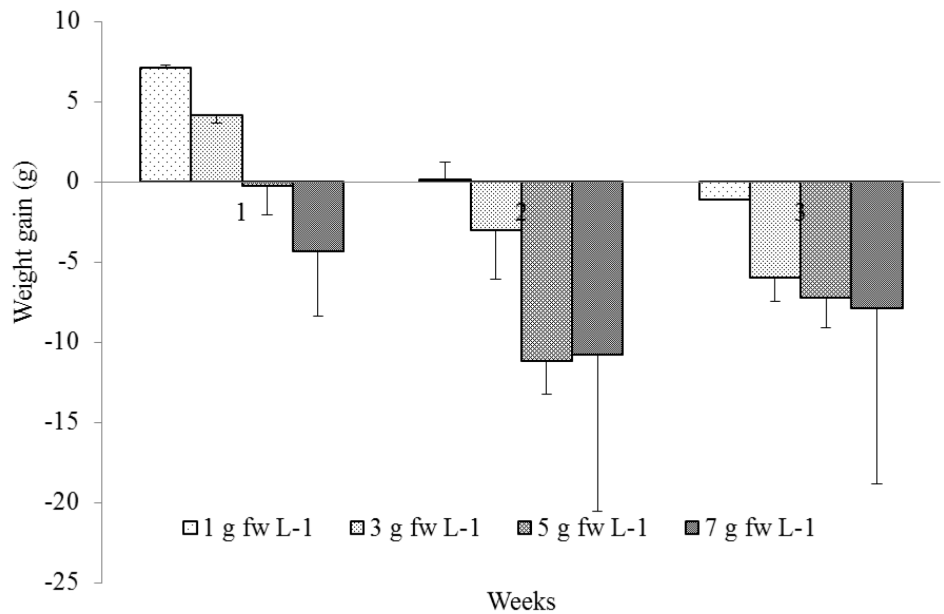

Fig 1. Weight gain (g) of *Spirogyra spp.* was reared in different stocking densities.

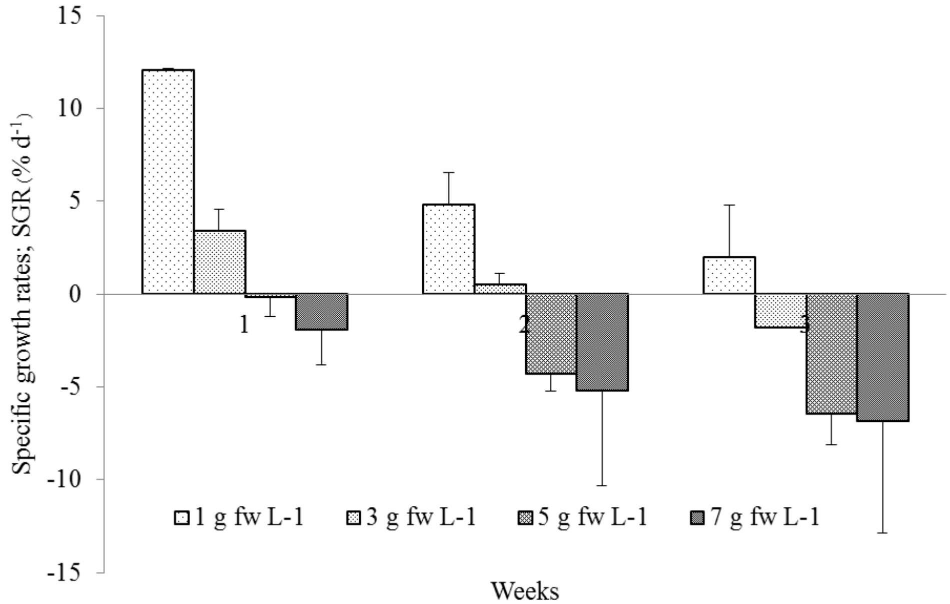

Fig 2. Specific growth rate (%d^{-1}) of *Spirogyra spp.* was reared in different stocking densities.

The first week of experiment, the effect of stocking densities to growth of *Spirogyra spp.* showed statistically significant differences in weight gain (F = 9.668; $p<0.05$) and specific growth rate (F=51.792; $p<0.05$) between treatments. Weight gain of algae were reared at 1 g fw L^{-1} was significantly higher than 0 (control treatment), 5 and 7 g fw L^{-1} (7.09±0.21 g) but no significant different when compared with 3 g fw L^{-1}. For the specific growth rate of algae were cultivated at 1 g fw L^{-1} showed significantly higher than other treatments (12.05±0.11 % d^{-1}) (Table 1).

Table 1. Weight gain (g) and specific growth rate (% d^{-1}) of *Spirogyra spp.* was grown in different stocking densities in the first week of experiment.

Parameters	**stocking density levels (g fw L^{-1})**				
	0	**1**	**3**	**5**	**7**
Weight gain (g)	0^{bc}	$7.09±0.21^{a}$	$4.14±0.50^{ab}$	$-0.25±1.80^{bc}$	$-4.32±4.06^{c}$
Specific growth rate (% d^{-1})	0^{c}	$12.05±0.11^{a}$	$3.37±1.23^{b}$	$-0.17±1.03^{c}$	$-1.95±1.87^{c}$

Mean±S.D. in the same row carrying different superscripts were significant different ($p<0.05$).

The second week of experiment, the effect of stocking densities to growth of *Spirogyra spp.* still showed statistically significant differences in weight gain ($F = 4.337$; $p<0.05$) and specific growth rate ($F=8.090$; $p<0.05$) between treatments. Weight gain of algae were reared at 1 g fw L^{-1} was significantly higher than 5 and 7 g fw L^{-1} (0.16±1.04 g) but no significant different when compared with 0 (control treatment) and 3 g fw L^{-1}. For the specific growth rate of algae were cultivated at 1 g fw L^{-1} showed significantly higher than 0 (control treatment), 5 and 7 g fw L^{-1} (4.83±1.75 % d^{-1}) but no significant different when compared with 3 g fw L^{-1} (Table 2).

Table 2. Weight gain (g) and specific growth rate (% d^{-1}) of *Spirogyra spp.* was grown in different stocking densities in the second week of experiment.

Parameters	**stocking density levels (g fw L^{-1})**				
	0	**1**	**3**	**5**	**7**
Weight gain (g)	0^{a}	$0.16±1.04^{a}$	$-3.03±3.05^{ab}$	$-11.17±2.08^{b}$	$-10.77±9.74^{b}$
Specific growth rate (% d^{-1})	0^{bc}	$4.83±1.75^{a}$	$0.48±0.63^{ab}$	$-4.29±0.90^{cd}$	$-5.18±5.10^{d}$

Mean±S.D. in the same row carrying different superscripts were significant different ($p<0.05$).

The third week of experiment, the *Spirogyra spp.* was reared in different stocking densities revealed negative of weight gain and no significant differences in weight gain ($F = 1.544$; $p>0.05$) between treatments but the specific growth rate of *Spirogyra spp.* showed statistically significant differences ($F=4.906$; $p<0.05$) between treatments. The specific growth rate of algae were grown at 1 g fw L^{-1} was significantly higher than 5 and 7 g fw L^{-1} (1.99±2.79 % d^{-1}) but no significant different when compared with 0 (control treatment) and 3 g fw L^{-1} (Table 3).

Table 3. Weight gain (g) and specific growth rate (% d^{-1}) of *Spirogyra spp.* was grown in different stocking densities in the third week of experiment.

Parameters	**stocking density levels (g fw L^{-1})**				
	0	**1**	**3**	**5**	**7**
Weight gain (g)	0	-1.13±0.76	-5.95±1.47	-7.19±1.90	-7.86±10.97
Specific growth rate (% d^{-1})	0^{a}	$1.99±2.79^{a}$	$-1.81±0.04^{ab}$	$-6.44±1.64^{b}$	$-6.83±6.02^{b}$

Mean±S.D. in the same row carrying different superscripts were significant different ($p<0.05$).

The end of experiment, the effect of different stocking densities as 0 (control treatment), 1, 3, 5 and 7 g fw L^{-1} to growth of *Spirogyra spp.* showed statistically significant differences in average weight gain ($F = 5.193$; $p<0.05$) and average specific growth rate ($F=12.603$; $p<0.05$) between treatments. The average weight gain of algae were grown at 1 g fw L^{-1} was higher than other treatments (1.41±3.62 g) but no significant different when compared with (control treatment) and 3 g fw L^{-1} of (Table 4). In the part of average specific growth rate, the algae were reared at 1 g fw L^{-1} was significantly higher than other treatments (5.57±4.56 % d^{-1}) (Table 5).

For the ability of *Spirogyra spp.* to ammonia nitrogen removal, the result found that the nitrogen absorbtion rate of algae was 0.045±0.003 mg-N L^{-1} g fw^{-1} d^{-1} at 1 g fw L^{-1} of stocking density level which the total ammonia nitrogen (mg-N L^{-1}) of water samples at the initial and the final of experiment were 0.89±0.30 mg-N L^{-1} and 0.63±0.22 mg-N L^{-1} respectively.

Table 4. Average weight gain (g) of *Spirogyra spp.* was grown in different stocking densities at the end of experiment.

Stocking density level (g fw L^{-1})	**Weeks**			**Average weight gain (g)**
	1	**2**	**3**	
0	0	0	0	0^{a}
1	7.09±0.21	0.16±1.04	-1.13±0.76	1.41 ± 3.62^{a}
3	4.14±0.50	-3.03±3.05	-5.95±1.47	-1.61 ± 4.88^{ab}
5	-0.25±1.80	-11.17±2.08	-7.19±1.90	-6.21 ± 5.07^{bc}
7	-4.32±4.06	-10.77±9.74	-7.86±10.97	-7.65 ± 8.11^{c}

Mean±S.D. in the same column carrying different superscripts were significant different ($p<0.05$).

Table 5. Average specific growth rate (% d^{-1}) of *Spirogyra spp.* was grown in different stocking densities at the end of experiment.

Stocking density level (g fw L^{-1})	**Weeks**			**Average specific growth rate (% d^{-1})**
	1	**2**	**3**	
0	0	0	0	0^{b}
1	12.05±0.11	4.83±1.75	1.99±2.79	5.57 ± 4.56^{a}
3	3.37±1.23	0.48±0.63	-1.81±0.04	0.68 ± 2.34^{b}
5	-0.17±1.03	-4.29±0.90	-6.44±1.64	-3.64 ± 2.96^{c}
7	-1.95±1.87	-5.18±5.10	-6.83±6.02	-4.66 ± 4.59^{c}

Mean±S.D. in the same column carrying different superscripts were significant different ($p<0.05$).

Discussions (with cited references)

The results from this study found that weight gain and specific growth rate of *Spirogyra spp.* were the highest in the first week of experiment and gradually decreased in the second and the third week respectively because of *Spirogyra spp.* can proliferate within 7 days after cultured and should harvest the productivity within 10 days and then the growth of algae will decrease (as cited in Uthumpa, 2011). The cultivation of *Spirogyra spp.* at different stocking densities affected to decline of weight gain and specific growth rate when stocking density increased which correspond with various studies. Nagler *et al*. (2003) studied the effects of fertilization treatment and stocking density on the growth and production of economic seaweed *Gracilaria parvispora* (Rhodophyta) in cage culture, their result showed that the maximal relative growth rate (RGR) of algae was 8.7% d^{-1} for 2 kg m^{-3} that was the lowest of stocking density in this studies and it declined to be 4% d^{-1} when stocking density increase at 8 kg m^{-3}. Likewise, the studies of Pereira *et al.* (2006) about the influence of stocking density, light and temperature on the growth, production and nutrient removal capacity of *Porphyra dioica* (Rhodophyta), their result found that the growth rate of algae decreased significantly with increasing stocking density. Moreover, Msuya (2013) researched the effects of stocking density and additional nutrients on growth of the commercially farmed seaweeds *Eucheuma denticulatum* and *Kappaphycus alvarezii* in Zanzibar Tanzania, found out that the specific growth rates (SGRs) of both species were significantly higher at the lower stocking density ($P<0.05$), whether the seaweeds had been fertilised or not. In addition, Kim *et al.* (2013) also studied about the effects of stocking density and temperature on nitrogen removal capacity of *Chondrus crispus* and *Palmaria palmate* (Rhodophyta), the result revealed that growth rate of both species were inversely dependent on stocking density at all temperature. A high stocking density can result in self-shading, therefore reducing photosynthetic rate of macroalgae that leads to reduce productivity and reduction of nutrient removal from water column (Kim *et al.*, 2013; Manríquez-Hernández, 2013). Beside, the cultivation under the high stocking density influences stressed of algae when competing for space and resources leading to low growth (Msuya, 2013).

The suitable stocking density to growth of *Spirogyra spp.* was 1 g fw L^{-1}. In general, the factors affect growing of algae consisted of various parameters such as nutrient, light intensity, water temperature, pH and salinity (Ren, 2014). However, within the experimental conduct, January until Febuary when is the suitable time of *Spirogyra spp.* appearance in natural water. Likewise, the experimental environment supports growing of *Spirogyra spp.* which water temperature ranged from 20.58±0.55 – 30.46±1.00ºC, pH ranged from 8.87±0.30 – 8.90±0.18, dissolved oxygen ranged from 4.73±0.22 – 6.11±0.64

mg L^{-1}, light intensity ranged from 3,006 – 18,188 lux and averaged of total ammonia nitrogen of water samples was 0.51±0.15 mg-N L^{-1}.

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References

Adrien, R. (2011). Characterization of freshwater algae from JKUAT and evaluation of its bioethanol and biodiesel potential. A thesis submitted in a partial fulfillment of the degree of master of science in chemistry in the Jomo Kenyatta University of agriculture and technology.

Atawodi, S.E, Uruawuike, S.C., Abdullahi, S.A., Bremauntz, P. and Krupek, R.A. (2015). Evaluation of *Spirogyra rhizobrachialis*, *Merismopedia elegans* and *Synedra spp.* as potential sources of proteases. Annual Review and Research in Biology 5 (2): p155.

Eshaq, F.S. (2010). *Spirogyra* biomass a renewable source for biofuel (bioethanol) production. International Journal of Engineering Science and Technology 2 (12): 7045-7054.

Feijoó, C., García, M.E., Momo, F. and Toja, J. (2002). Nutrient absorption by the submerged macrophyte *Egeria densa* Planch.: Effect of ammonium and phosphorus availability in the water column on growth and nutrient uptake. Limnetica 21 (1-2): 93-104.

Kim, J.K., Duston, J., Corey, P. and Garbary, D.J. (2013). Marine finfish effluent bioremediation: Effect of stocking density and temperature on nitrogen removal capacity of *Chondrus crispus* and *Palmaria palmate* (Rhodophyta). Aquaculture 414-415: 210-216.

Macchiavello, J. and Bulboa, C. (2014). Nutrient uptake efficiency of Gracilaria *chilensis* and *Ulva lactuca* in an IMTA system with the red abalone *Haliotis rufescens*. Latin American Journal of Aquatic Research 42(3): 523-533.

Mane, P.C. and Bhosle, A.B. (2012). Bioremoval of some metals by living algae *Spirogyra sp.* and *Spirulina sp.* from aqueous solution. International Journal of Environmental Research 6(2): 571-576.

Manríquez-Hernández, J.A. (2013). Interaction of irradiance and stocking density on nutrient uptake by red macroalgae implications for bioremediation of fish farm effluents. Submitted in partial fulfillment of the requirements for the degree of master of science, Dalhousie University.

Msuya, F.E. (2013). Effects of stocking density and additional nutrients on growth of the commercially farmed seaweeds *Eucheuma denticulatum* and *Kappaphycus alvarezii* in Zanzibar Tanzania. Tanzania Journal of Natural and Applied Sciences 4(1): 605-612.

Nagler, P.L., Glenn, E.P., Nelson, S.G. and Napolean, S. (2003). Effect of fertilization treatment and stocking density on the growth and production of the economic seaweed *Gracilaria parvispora* (Rhodophyta) in cage culture at Molokai, Hawaii. Aquaculture 219: 379-391.

Peerapornpisal, Y. (2008). Edible freshwater macroalgae in Northern Thailand research. Journal of Fisheries Technology Research 2(1): 178-189.

Peerapornpisal, Y. (2013). Freshwater algae in Thailand. Second edition, department of biology, faculty of science, Chiang Mai University, Chiang Mai.

Pereira, R., Yarish, C. and Sousa-Pinto, I. (2006). The influence of stocking density, light and temperature on the gowth, production and nutrient removal capacity of *Porphyra dioica* (Bangiales, Rhodophyta). Aquaculture 252: 66-78.

Punyoyai, T. (2008). Antioxidant activity of Tao, *Spirogyra neglecta* (Hassall) KÜtzing. M.S. thesis, department of biology, faculty of science, Chiang Mai University, Chiang Mai.

Ramaraj, R., Unpaprom, Y., Whangchai, N. and Dussadee, N. (2015). Culture of macroalgae *Spirogyra ellipsospora* for long-term experiments, stock maintenance and biogas production. Emergent Life Science Research 1(1): 38-45.

Ren, T. (2014). Primary factors affecting growth of microalgae optimal light exposure duration and frequency. A thesis submitted to the graduate in partial fulfillment of the requirement for the degree of master of science (environmental engineering), Iowa State University, Ames, Iowa.

Sirirustananun, N. and Chanartaeparporn, P. (2015). Nutrient contents and growth of *Spirogyra spp.* in water resources of Phetchabun province. Journal of Agricultural Research and Extension 32(1): 58-66.

Thiamdao, S. and Peerapornpisal, Y. (2009). Diversity of edible freshwater macroalgae in Mekong and Nan rivers. Journal of Fisheries Technology Research 3(1): 115-124.

Thiamdao, S and Peerapornpisal, Y. (2011). Morphological observation of *Spirogyra ellipsospora* Transeau, an edible freshwater macroalgae. Journal of the Microscopy Society of Thailand 4(2): 94-97.

Tipnee, S., Ramaraj, R. and Unpaporm, Y. (2015). Nutritional evaluation of edible freshwater green macroalgae *Spirogyra varians.* Emergent Life Science Research 1(2): 1-7.

Uthumpa, C. (2011). A comparative study of *Spirogyra sp.* yield when cultured in plastic buckets with different ratio of inorganic fertilizer (formula 10:25:20). A thesis submitted to the graduate in partial fulfillment of the requirement for the degree of master of science (teaching science), Chiang Rai Rajabhat University, Chiang Rai.

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