Productivity, biochemical composition and biofiltering performance of agarophytic seaweed, *Gelidium elegans* (Red algae) grown in shrimp hatchery effluents in Malaysia

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Abstract

Aquaculture systems may release large amounts of nutrients into the marine ecosystem that can lead to eutrophication. Many seaweed species are efficient in reducing the nutrient concentration in aquaculture waste. In this study, the agarophyte *Gelidium elegans* originating from Korea was investigated for bioremediation of tiger shrimp (*Penaeus monodon*) broodstock effluent (SBE) in a shrimp hatchery in Malaysia. The *G. elegans* was cultivated in SBE using a semi-continuous culture system to evaluate the effects of elevated nutrients on the biomass, growth rate, quality and quantity of agar, pigment content and biochemical composition of seaweed. In the treatment containing *G. elegans*, pollutant nutrient concentration was reduced by 100%, and by 78.7%, 47.9%, and 19.6% for ammoniacal-nitrogen, nitrite, nitrate, and orthophosphate, respectively. The seaweed grew well (1.4 ±0.3% d^{-1}) in SBE and produced appreciable amounts of protein (3.8% DW), carbohydrate (41.4 % DW) and agar (25.9% DW), that were significantly enhanced when cultured in the SBE compared to control. The photosynthetic performance of *G. elegans* assessment using a Diving-PAM showed the healthy condition of the grown seaweed without any indication of stress in shrimp hatchery effluent. Results showed that *G. elegans* has biofiltering and bioremediation potential for the shrimp hatchery effluent.

Keywords: *Gelidium elegans*, Red seaweed, Bioremediation, Pulp, Agar, Biochemical, Malaysia

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Introduction

Aquaculture and capture fisheries supplied the world fish production with about 156.2 million tonnes in 2011, of which 40.1% (62.7 million tonnes) was attributed to marine and inland aquaculture (FAO, 2013). In 2011, Asian countries produced 88.5% (55.5 mt) of world farmed fish (FAO, 2013). The rapid growth of aquaculture is accompanied by increased discharge of nutrient-rich wastewaters into rivers and coastal waters, leading to eutrophication, and deterioration of water quality (Shpigel and Neori, 2007; Marinho-Soriano *et al*., 2009a). Un-optimised feeding in aquaculture produces large amounts of dissolved inorganic nitrogen and phosphorus. The challenge in environmental management of aquaculture systems is to minimise the nutrient output as well as to remove them through conversion into useful products (Shpigel and Neori, 2007). Bioremediation using integrated aquaculture systems with bivalves as suspension feeders, microalgae and seaweeds as nutrient scrubbers and oxygenators offers a good alternative and cost-effective, conventional treatment systems (Shpigel and Neori, 2007). Seaweeds may play an important role in improving water quality and contribute to sustainable aquaculture (Copertino *et al.*, 2009; Marinho-Soriano *et al*., 2009b). Seaweeds can remove excessive nutrients, organic pollutants and heavy metals through bioconcentration and detoxification mechanisms, while improving dissolved oxygen levels and removing up to 90 % nutrients like ammonia from fish ponds (Shpigel and Neori, 2007). Seaweeds such as *Gracilaria birdiae*, *G. edulis*, *G. lemaneiformis* and *Ulva lactuca* can efficiently remove dissolved nutrients from fish and shrimp effluents and produce a biomass with useful products such as agar, food and feed for high valued aquaculture organisms, fertilizer and bioethanol (Neori *et al*., 2004; Copertino *et al*., 2009; Marinho-Soriano *et al*., 2009a; Kim *et al*., 2010; Abreu *et al*., 2011). In Malaysia, shrimp farming of *Penaeus monodon* provides the bulk of the revenue to the aquaculture industry, making Malaysia the world's 16th largest aquaculture producer (FAO, 2013). The integration of seaweed with shrimp may provide an economically feasible solution to improve water quality and to provide an extra harvestable product, thereby producing a sustainable shrimp industry. Some species of seaweed such as *Laminaria* spp. and *Macrocystis* spp. have high primary productivity of≥ 3000 g C m^{-2} y^{-1} (Gao and McKinley, 1994) and the harvest of seaweed biomass may result in carbon drawdown, indicating high potential for CO_2 remediation (Chung *et al*., 2011).

The species of *Gelidium* are the best raw materials for bacteriological agar and agarose in the world (McHugh, 2003) and has been cultured in Korea (Melo, 1998), China (Fei and Huang, 1991), Spain and Portugal (Salinas, 1991) and two general cultivation systems have been used for *Gelidium*;

mariculture in the sea or culture in tanks both utilising vegetative fragments (Titlyanov *et al*., 2006, Friedlander, 2008). Some species of *Gelidium* such as *G. amansii* and *G. sesquipedale* were used as biofilters for nitrogen removal from fishpond effluents (Salinas, 1991; Liu *et al*., 2004) and showed > 70% removal efficiency for NH_4^+.

G. elegans Kützing from Korea, which has already been reported as *G. amansii* (Kim *et al*., 2012) besides its high quality agar content has been used as a source of fine fibre for high quality paper (Seo *et al*., 2010) as well as a good source for bioethanol (Kim *et al*., 2010). The University of Malaya in collaboration with the Fisheries Department and Pegasus International, Korea, is investigating to introduce and cultivate *G. elegans* for the mentioned proposes. Many shrimp farms in Malaysia operate their own hatchery where broodstock are maintained for continuous supply of pathogen-free larvae. The shrimp broodstock effluent (SBE) has to be treated before discharge into the sea. Therefore in this study, we assessed the viability of *G. elegans* for culture in tiger shrimp (*P. monodon*) broodstock effluents. The objective of the study was to improve the quality of the shrimp broodstock effluent for discharge, and produce a useful seaweed biomass. In this paper, we monitored the growth of *G. elegans* in SBE, its ability to remove nutrients and the biochemical compounds of the species like agar, protein, carbohydrate and pigments which may allow the resulting seaweed biomass to serve as a bioresource for feed, pulp or biofuel production.

Materials and methods

The study was conducted in a shrimp hatchery using a semi-continuous culture system in Kuala Selangor (03º17′38″ N, 101º17′8″ E) in Malaysia from 6th April 2012 for 6 months. The *G. elegans* used in this study was from a uniculture originating from South Korea and was quarantined for three months in the nursery in the University of Malaya, with continual cleaning of the seaweed branches to ensure removal of any epiphytic organisms. Seaweed stocks after acclimatisation to local conditions and generation new branches were transferred to the tiger shrimp hatchery in Kuala Selangor which was stored in three 200 L rectangular, flat-bottomed plastic tanks containing filtered natural seawater with continuous aeration and daily water exchange (30 %) for two weeks until the start of the bioremediation experiment. During the acclimatisation period, the seaweed stocks were maintained free of epiphytes and necrotic portions. After final acclimatisation, the healthy plants which were firm in texture, dark red in colour, and clear of epiphytes, were selected and transferred to the treatment tanks placed under a shelter open on all sides but roofed with transparent polycarbonate sheets for the bioremediation experiments.

The shrimp effluent used in the experiment was provided from a 3000L cement pond containing about 70 broodstock shrimps of *P. monodon* with an average weight of 150 g which was maintained with 30% daily water exchange. The discharge effluent was used for the experiment.

The experimental design for seaweed cultivation consisted of four water treatments in triplicates. Treatments were: (i) Tanks with only seawater (SW control); (ii) *G. elegans* in seawater (SW+*G. elegans*); (iii) shrimp broodstock effluent only (SBE control); (iv) *G. elegans* in SBE (SBE+*G. elegans*). Twelve (4 treatments; 3 replicates) 150 L rectangular plastic tanks (70 cm width×125 cm length×30 cm depth) with flat bottoms, were used in the experiment. 450 g (3 g L^{-1}) fresh weight (FW) of *G. elegans* was placed in each tank for the treatments with seaweed. The tank contents were continuously aerated using air-pumps and air stones submerged in the water at the tank bottom. Every two days, 50 L (30%) of the tank content was replaced with fresh seawater or SBE. This semi-continuous culture was continued for 12 days.

The biomass FW and growth rate of the *G. elegans* were measured at two-day intervals for 12 days. The entire biomass of each tank was taken out and spread in a plastic basket for the excess water to drain off before weighing with the Sartorius BL610 (±0.01g) balance. The relative growth rate (RGR) was calculated using the following equation (Phang *et al*., 1996) as Eq. (1):

$$RGR\ (\%\ d^{-1}) = [(W_t - W_i) / (W_i * \Delta t)]*100 \quad (1)$$

Where W_i is the initial fresh weight, W_t is the fresh weight on day t, Δt is the time interval.

The photosynthetic performance of *G.elegans* was assessed fluorometrically by using a Diving-PAM (Pulse Amplitude Modulation) fluorometer (Walz, Germany) before and after the seaweed was grown in SBE and seawater. Maximum quantum yield (F_v/F_m) was used to indicate the healthy physiological state of the seaweed. It is an indicator of stress (Figueroa *et al*., 2009), and is defined as:

$$F_v/F_m = (F_m - F_0)/F_m$$

where F_v is the variable fluorescence measured as the difference between maximum (F_m) and minimum (F_0) fluorescence in dark adapted seaweed. The seaweeds were dark adapted for 15 min prior to the maximum quantum yield determination (Gao *et al*., 2004) using dark leaf clips. Three replicates were measured from each tank. Photosynthetic efficiency was determined from the initial slope (α) of rapid light curve (RLC).

During the experiment, water quality and environmental variables were monitored every 48h. The physicochemical parameters such as temperature, salinity, dissolved oxygen concentration (DO) and pH were measured *in situ* by a YSI (Model 85) multi-parameter probe. Irradiance was measured above the surface of the tank water using the LI-COR (LI-250A) light meter. Water samples for

determination of water quality were taken in the morning at 11.30 am every two days before and after exchange of one-third (50 L) of tank water during the culture period. Water samples were stored in polyethylene bottles, transported in the ice chest (10 ºC) to the laboratory for analysis. The concentration of ammoniacal nitrogen (NH_3-N) was measured by the salicylate method, nitrite (NO_2-N) by diazotization, nitrate (NO_3-N) by cadmium reduction, orthophosphate (PO_4-P) by the ascorbic acid method, and chemical oxygen demand (COD) by reactor digestion method using the HACH spectrophotometer (Model DR/2500) (Clesceri *et al*., 1998). The other parameters such as total suspended solids (TSS), total solids (TS), total volatile solids (TVS) and total dissolved solids (TDS) were analyzed in the laboratory using various analytical methods according to the standard methods for examination of water and wastewater (Clesceri *et al*., 1998).

The removal efficiency of nutrients in each tank was estimated by subtracting the initial (inflow) and final (outflow) concentrations of nutrients every two days (before and after exchange of water). Percentage removal efficiency (RE %) in each tank was calculated using methods described by Hayashi *et al*. (2008) as Eq. (2):

$$RE\ \% = [(Inflow - Outflow)/Inflow] \times 100 \quad (2)$$

Where: *Inflow* = nutrient concentration (mg L^{-1}) after replacement water content of each tank with 30% of fresh seawater or SBE; *Outflow* = nutrient concentration (mg L^{-1}) before water replacement in each tank.

Biochemical composition was analysed using the seaweed samples that were cleaned of epifauna and epiphytes by washing in diluted seawater, blot-dried and weighed. Dry weight (DW) was calculated by weight loss after drying in oven at 70 ^{o}C for 72 h and ash content was calculated from weight remaining after incineration of dry matter for 6 h at 550 $^{\circ}$C in a muffle furnace. Protein (Bradford, 1976), carbohydrate (Dubois *et al*., 1956), chlorophyll *a*, carotenoids and phycobiliproteins (Lim *et al*., 2010) and agar were determined on the first day (day 0) and last day (day 12) of the experiment. Carbohydrate, protein and agar content of *G. elegans* were analysed and expressed in percentage dry weight (% DW). The agar extraction was carried out using methods by Phang *et al*. (1996). Gel strength (g cm^{-2}), gelling and melting temperature (ºC) were tested by preparing a 1.5 % w/v agar solution according to methods described by Phang *et al*. (1996). Gel strength was measured at ambient temperature using a Nikkansui Gel Strength tester (Kiya Seisakusho Tokyo Japan).

Statistical analysis

The growth, variation in nutrient concentration and nutrient removal of *G. elegans* in different water (four levels) and time (seven levels)

treatments during the culture period (12 days) were compared using a Split plot design in time arranged in a randomized complete block design (RCBD) with three replications after assessing normality and homogeneity using Kolmogorov- Smirnov and Levene's tests, respectively. The water treatments (four levels) were allocated to the main plots. Time treatments (seven levels) were distributed within the sub-plots in each of the water treatments. Tukey's-HSD comparisons were applied to determine statistically significant differences (p<0.05) among time (day) and water treatments. Independent sample t-tests were utilized to determine the effect of treatments on quality and quantity of agar, protein, carbohydrate and pigment content on the last day of the experiment. One-way ANOVA was used for analyses of physicochemical parameters in different treatments. A significance level of 95 % ($\alpha = 0.05$) was set for all the tests. The statistical analyses were carried out using SPSS software, version 15 (SPSS Inc., USA).

Results

The biomass (g L^{-1} FW) of *G. elegans* cultured in various water treatments (SBE+*G. elegans* & SW+*G. elegans*) over time (day) are shown in Fig. 1. In SBE, the *Gelidium* biomass ranged from 3.00 to 3.70 g L^{-1}, with a mean final biomass of 3.55±0.14 g L^{-1} FW (18% increase) on day 12. In SW, the seaweed biomass ranged from 3.00 to 3.06 g L^{-1}, with a mean final biomass of 3.02 ±0.00 g L^{-1} FW (0.6 % increase) at day 12. ANOVA showed the biomass of *G. elegans* was significantly influenced by both water treatment and time (p<0.01). Throughout the culture, significantly higher biomass of *G. elegans* was obtained in SBE+*G. elegans* than in SW+*G. elegans* (p<0.01). The highest average biomass (3.55±0.14 g L^{-1}) was obtained on day 12 in SBE + *G. elegans*.

The relative growth rates (RGR) of *G. elegans* cultured in various treatments are shown in Fig.1. The water treatment and time produced significant difference (p<0.01) in the RGR of *G. elegans*. In general, *G. elegans* grown in SBE exhibited significantly (p<0.05) higher mean RGR compared to the *G. elegans* in seawater treatments (Fig. 1). In SBE, the RGR (%d^{-1}) ranged from 0.13–1.76, with a mean of 1.40±0.34 %d^{-1} on day 12. In seawater treatments the RGR (%d^{-1}) ranged from 0.04–1.19, with a mean of 0.16 ±0.04 %d^{-1} on day 12 .

The characteristics of the seawater and SBE in inflow are shown in Table 1. The mean and the range of physico-chemical parameters of the outflow water during 12 days are shown in Table 2. The irradiance throughout the experiment was in the average of 91.5±21.0 µmol photons $m^{-2}s^{-1}$. Maximal pH values were measured in the tanks containing *G. elegans*. Mean pH fluctuated between 7.9±0.3 and 8.3±0.1 with significant difference for the four treatments during 12 days (Table 2). The salinity fluctuated between 24.6 and 29.3 (‰). Mean

dissolved oxygen (mgL^{-1}) concentrations fluctuated between 2.6±0.4 and 3.1±1.0 with significant differences in SBE treatment and other treatments (Table 2). ANOVA test showed significant differences (Tukey HSD, $p<0.05$) for pH and salinity during 12 days of culture (Table 2).

Table 1: The mean physico-chemical characteristics of seawater and shrimp broodstock effluent in inflow at the start of experiment and every alternate day during 12 days .

Parameters	SBE		SW Day 0
	Day 0	Mean (day 0-12)	
NH_3-N (mg L^{-1})	0.04±0.01	0.05±0.02	0.01±0.00
NO_3-N (mg L^{-1})	18.10±0.00	10.52±4.41	0.13±0.00
NO_2-N (mg L^{-1})	8.10±0.00	3.93±2.25	0.01±0.00
PO_4-P (mg L^{-1})	1.92±0.01	1.28±0.44	0.36±0.01
COD (mg L^{-1})	233.30±30.50	466±289	706.60±32.15
Salinity (‰)	27.50±0.20	27.4±1.00	27.60±0.00
pH	7.30±0.01	7.7 ±0.24	7.32±0.00
TS (mg L^{-1})	35462±137	33124±2034	32128±12
TSS (mg L^{-1})	216±13	207±15	165±9
TVS (mg L^{-1})	6094±190	5764±290	5144±17
TDS (mg L^{-1})	35246±124	32917±2025.60	31963±98
Total carbon (mg L^{-1}) (COD x 12/32)	87±6	174±12	211±2
Total nitrogen (mg L^{-1}) (NH_3-N + NO_3 + NO_2)	26.24±2.34	14.50 ±4.53	0.15±0.01
C: N: P	46:14:1	136:11:1	587:0.4:1

Each value represents a mean ±SD

Table 2: The mean physico-chemical characteristics of water in tanks with *Gelidium elegans* and without *G. elegans* (Control) in the outflow in a semi-continuous culture system during 12 days (every alternate day).

Parameters	Treatments			
	SBE+*G. elegans*	SBE	SW+*G. elegans*	SW
Water temperature (°C)	27.90±1.14[a]	28.90±1.84[a]	27.9 ±0.96[a]	28.20±1.43[a]
pH	8.03±0.42[c]	8.20±0.09[b]	8.31±0.12[a]	7.86±0.30[d]
Salinity(ppt)	28.07±0.43[a]	26.80±1.14[b]	26.96±0.64[b]	27.97±0.59[a]
DO (mgL^{-1})	2.63±0.37[b]	3.06±1.01[a]	2.71±0.29[b]	2.71±0.85[b]

Values in each row bearing different superscripts are significantly different based on Tukey HSD test at $p<0.05$ between treatment (SBE=Shrimp broodstock effluent; SBE+*G. elegans*=Shrimp broodstock effluent and *G. elegans*; SW= Seawater; SW+*G. elegans*= Seawater and *G. elegans*). Each value represents a mean ±SD (n=21).

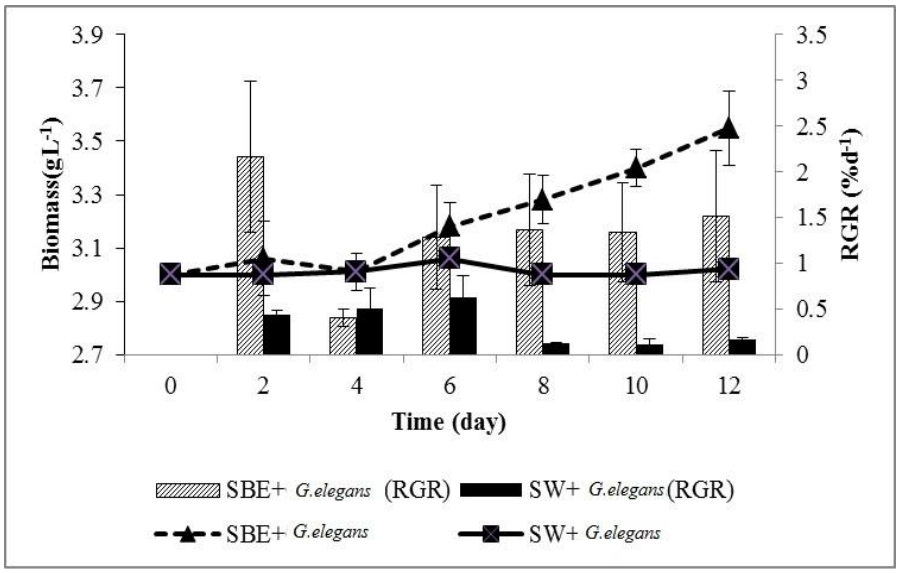

Figure 1: Biomass (line; gL^{-1}) and relative growth rate (bar; % d^{-1}) of *Gelidium elegans* cultivated in shrimp broodstock effluent (SBE) and seawater (SW) using a semi-continuous system in the hatchery. Each value represents a mean ±S.D. (n=3).

Table 3 gives the concentration of different nutrients in the four treatments (SBE+*G. elegans*, SBE, SW+*G. elegans*, SW) during the 12 days of culture. The presence of *G. elegans* in both SBE+*G. elegans* and SW+*G. elegans*, resulted in significant (p<0.01) decrease in all nutrient concentrations after 12 days compared to the control tanks (SBE, SW). The Tukey - HSD test (p<0.01) indicated that water treatment and time (day), and the interaction between water treatment and time, had significant (p<0.05) effect on concentrations of NH_3-N, NO_3-N, NO_2-N and PO_4-P during the cultivation (Tables 3, 4).

Table 3: Concentration of nutrients in outflow of different treatments every alternate day during 12 days in a semi-continuous culture system.

Nutrient	Treatment	Day						
		0	2	4	6	8	10	12
NH_3-N (mg L^{-1})	SBE+ *G. elegans*	0.05 ±0.01^{a}	0.01 ±0.00^{bc}	0.02 ±0.00^{b}	0.01 ±0.00^{bc}	nd^{c}	nd^{c}	nd^{c}
	SBE	0.04 ±0.01^{f}	0.10 ±0.00^{c}	0.26 ±0.01^{a}	0.18 ±0.00^{b}	0.08 ±0.00^{d}	0.06 ±0.01^{e}	0.06 ±0.01^{ef}
	SW+ *G. elegans*	0.00 ±0.00^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
	SW	0.01 ±0.00^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
NO_3-N (mg L^{-1})	SBE+ *G. elegans*	18.80 ±1.13^{a}	14.20 ±0.90^{b}	10.50 ±0.45^{c}	9.20 ±0.08^{dc}	8.50 ±0.70^{d}	4.20 ±0.35^{e}	3.00 ±0.45^{e}
	SBE	18.10 ±0.00^{a}	18.20 ±0.30^{a}	8.40 ±0.15^{d}	11.50 ±0.35^{b}	9.80 ±0.23^{c}	6.70 ±0.20^{e}	6.90 ±0.11^{e}
	SW+ *G. elegans*	0.06 ±0.00^{c}	0.47 ±0.06^{a}	0.16 ±0.06^{bc}	0.50 ±0.00^{a}	0.30 ±0.10^{b}	0.03 ±0.06^{c}	0.50 ±0.00^{a}
	SW	0.13 ±0.00^{e}	0.70 ±0.10^{ab}	0.83 ±0.05^{ab}	0.73 ±0.05^{ab}	0.90 ±0.10^{a}	0.30 ±0.00^{e}	0.63 ±0.05^{d}
NO_2-N (mg L^{-1})	SBE+ *G. elegans*	7.80 ±0.60^{a}	6.00 ±0.10^{b}	4.70 ±0.05^{c}	3.40 ±0.05^{d}	2.46 ±0.05^{e}	1.30 ±0.26^{f}	0.30 ±0.10^{g}

Table 3 continued:

	SBE	8.10 ±0.00^{a}	7.60 ±0.10^{b}	3.70 ±0.10^{c}	3.40 ±0.05^{d}	3.30 ±0.05^{d}	3.13 ±0.05^{e}	2.16 ±0.05^{f}
	SW+ *G. elegans*	0.01 ±0.00^{a}	0.00 ±0.00^{b}	0.00 ±0.00^{b}	0.00 ±0.00^{b}	0.00 ±0.00^{b}	0.00 ±0.00^{b}	0.00 ±0.00^{b}
	SW	0.01 ±0.00^{a}	0.01 ±0.00^{a}	0.00 ±0.00^{d}	0.01 ±0.00^{b}	0.01 ±0.00^{c}	0.00 ±0.00^{d}	0.00 ±0.00^{d}
	SBE+ *G. elegans*	1.90 ±0.09^{a}	1.90 ±0.28^{a}	1.40 ±0.09^{b}	1.25 ±0.02^{bc}	1.15 ±0.01^{bc}	0.95 ±0.03^{c}	0.97 ±0.10^{c}
PO_4-P (mg L^{-1})	SBE	1.90 ±0.01^{b}	1.99 ±0.00^{a}	0. 90 ±0.02^{f}	1.50 ±0.01^{c}	1.34 ±0.01^{d}	1.05 ±0.01^{e}	1.03 ±0.00^{e}
	SW+ *G. elegans*	0.30 ±0.01^{a}	0.07 ±0.00^{e}	0.08 ±0.01^{cd}	0.08 ±0.00^{cd}	0.05 ±0.01^{f}	0.140 ±0.01^{b}	0.09 ±0.01^{c}
	SW	0.36 ±0.01^{a}	0.22 ±0.01^{b}	0.08 ±0.01^{de}	0.23 ±0.01^{b}	0.14 ±0.00^{c}	0.06 ±0.01^{e}	0.09 ±0.01^{d}

Values in each row bearing different superscripts are significantly different based on Tukey HSD test at $p<0.05$ by effect of day; nd= not detectable. SBE=Shrimp broodstock effluent; SBE+*G. elegans* = Shrimp broodstock effluent and *G. elegans*; SW = Seawater; SW+*G. elegans*=Seawater and *G. elegans.* Each value represents a mean ±SD (n=3)

Table 4: Mean squares from analysis of variance of nutrients concentration in outflow under different treatments during 12 days.

Source of variance	df	Nutrients			
		NH_3-N	NO_3-N	NO_2-N	PO_4-P
Replication	2	2.190ns	0.055ns	0.025ns	0.002ns
Water Treatment(A)	3	0.61**	730.527**	120.299**	10.705**
R*A	6	2.570	0.036	0.025	0.002
Time(B)	6	0.005**	76.000**	18.078**	0.671**
A*B	18	0.005**	29.042**	6.593**	0.152**
R*B	12	1.320	0.133	0.019	0.004
Error	36	1.980	0.146	0.018	0.004

(**, significant difference at $p<0.01$; ns: not significant)

Removal efficiency (%) of nutrients in treatment containing *G. elegans* (SBE+*G. elegans* , SW+*G. elegans*) is presented for every 2 days during 12 days (Figs. 2, 3, 4, 5).

The NH_3-N concentration and removal efficiency (RE %) of all treatments are presented in Table 3 and Fig. 2. Ammoniacal-nitrogen was non-detectable in treatment SBE+*G. elegans* after 6 days, while in SBE (control) treatment, NH_3-N concentration increased by 46.5 %. In the SBE+*G. elegans*, the highest % RE (100 %) was calculated on day 10.

The NO_3-N concentrations and removal efficiency of nitrate in all treatments over 12 days are presented in Table 3 and Fig. 3. The nitrate concentration decreased significantly ($p<0.01$) in treatments SBE+*G. elegans* (from 18.8±1.1 to 3.0±0.4) and SBE (from 18.1±0.0 to 6.9±0.1) on day 12, with highest nitrate removal (48 %) in SBE + *G. elegans* tanks on day 10.

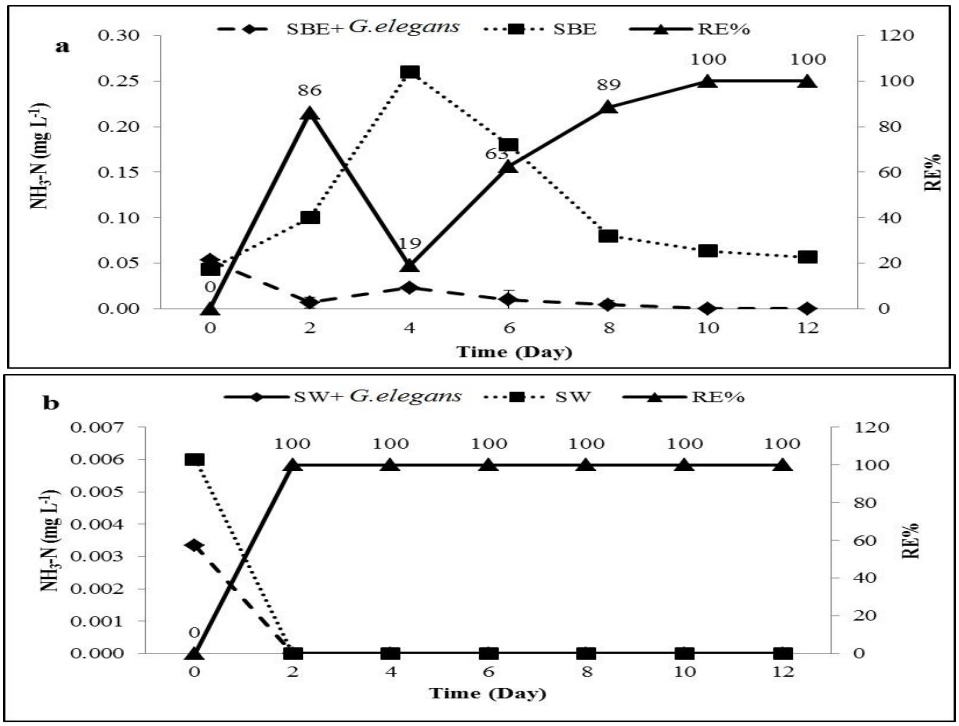

Figure 2: Concentration of NH_3-N in outflow of control and seaweed tanks during 12days in different treatments: a) in SBE (SBE+ *Gelidium elegans* and SBE), b) in seawater (SW+ *G. elegans* and SW). Removal efficiency of NH_3-N by *G. elegans* in seaweed tank presented as percentage (%RE).

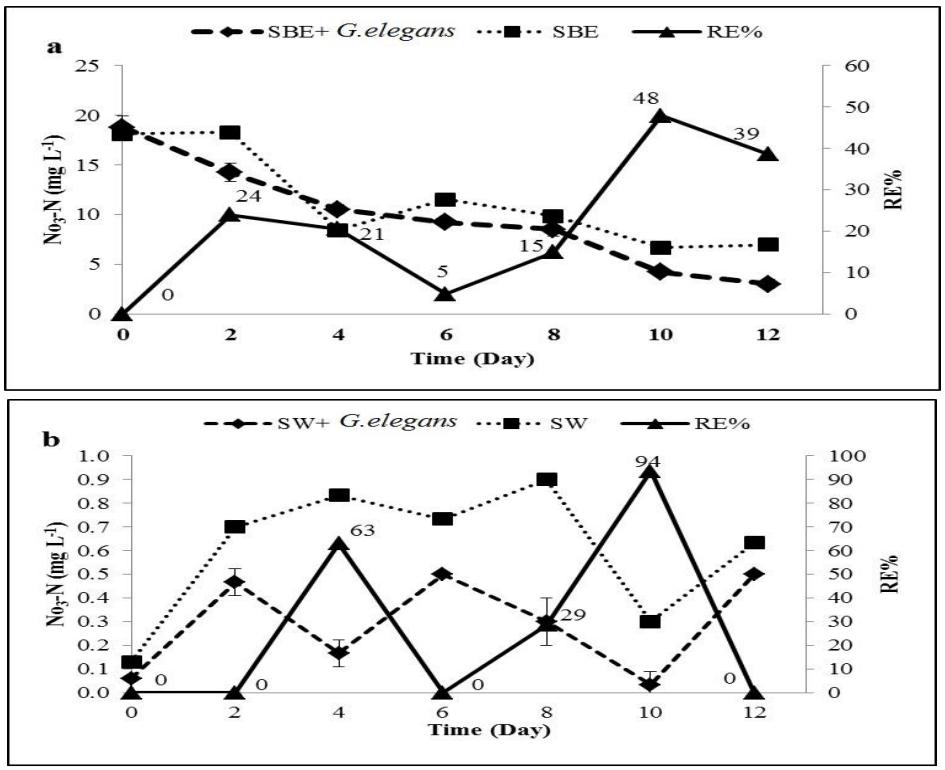

Figure 3: Concentration of NO_3-N in outflow of control and seaweed tanks during 12 days in different treatments: a) in SBE (SBE+ *Gelidium elegans* and SBE), b) in seawater (SW+ *G. elegans* and SW). Removal efficiency of NO_3-N by *G. elegans* in seaweed tank presented as percentage (%RE).

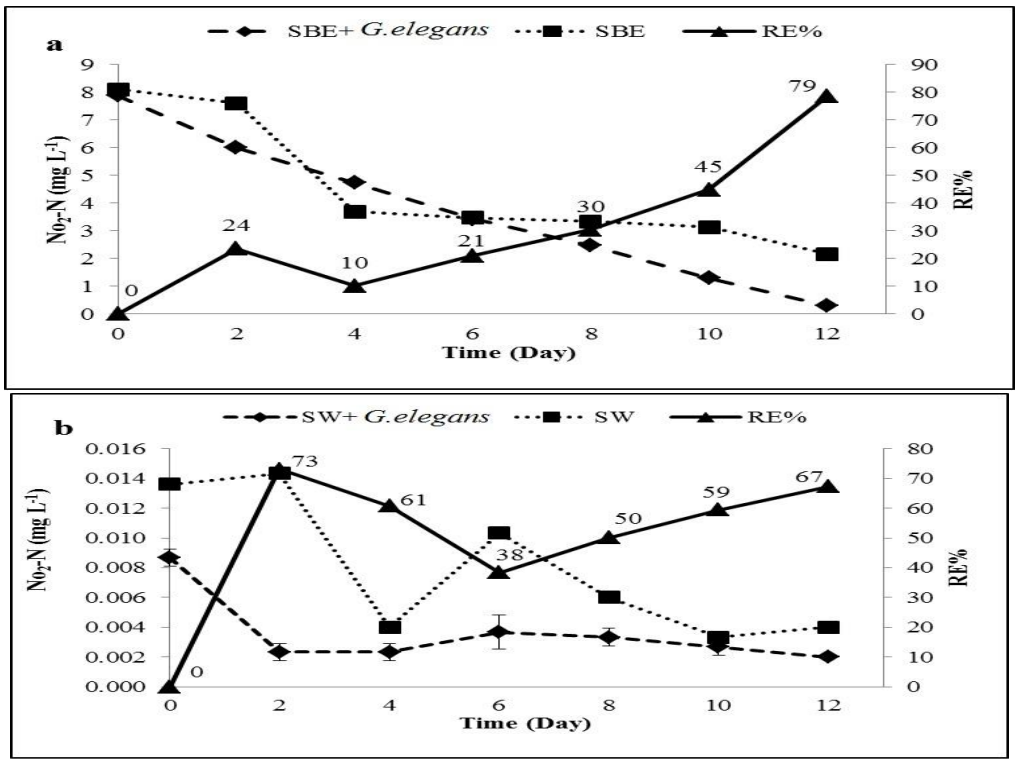

Figure 4: Concentration of NO_2-N in outflow of control and seaweed tanks during 12 days in different treatments: a) inSBE (SBE+ *Gelidium elegans* and SBE), b) in seawater (SW+ *G. elegans* and SW). Removal efficiency of NO_2-N by *G. elegans* in seaweed tank presented as percentage (%RE).

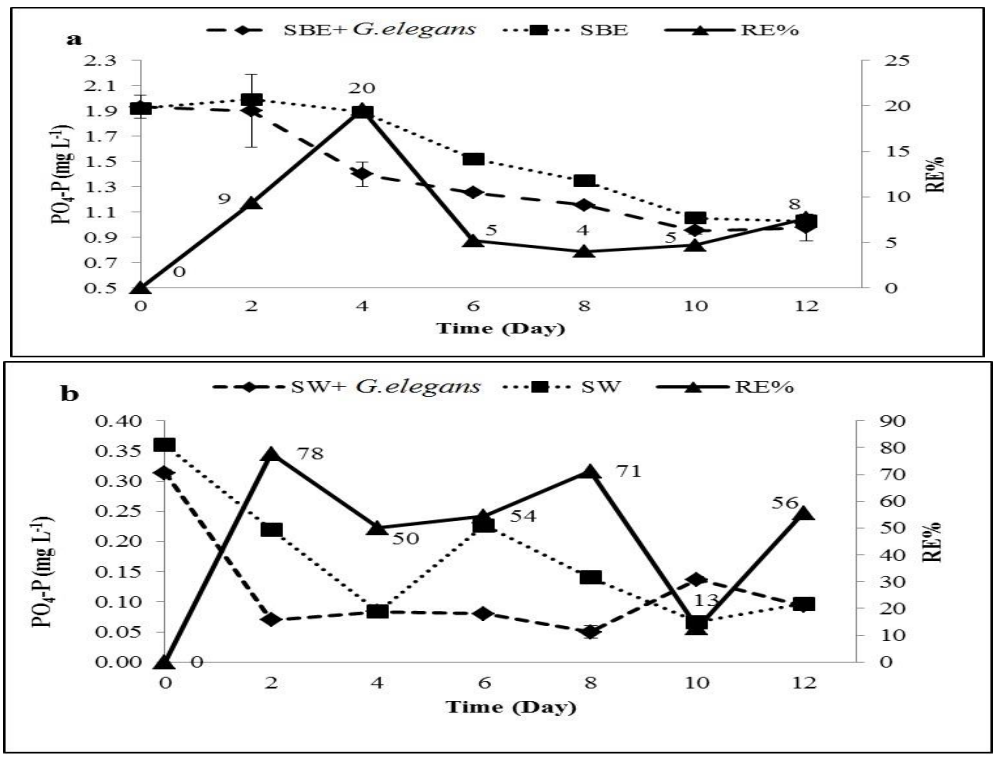

Figure 5: Concentration of PO_4-P in control and seaweed tanks during 12 days in different treatments: a) in SBE (SBE+ *Gelidium elegans* and SBE), b) in seawater (SW+ *G. elegans* and SW). Removal efficiency of PO_4-Pby *G. elegans*in seaweed tank presented as percentage (%RE).

There was a high variation in nitrite concentrations and nitrite removal efficiency (RE%) under different treatments and time (Table 3, Fig. 4). The ortho-phosphate (PO_4-P) concentrations and removal efficiency (RE%) during 12 days are presented in Table 3 and Fig. 5. The PO_4-P concentration significantly (p<0.01) decreased over the culture period in all treatments. Tukey HSD analysis showed that the mean concentrations of PO_4-P over time in treatments with *G. elegans* (SBE+*G. elegans*, SW+*G. elegans*) were significantly (p<0.01) lower than that in the control tanks (SBE, SW). The ortho-phosphate removal by *G. elegans* in treatment SBE+*G. elegans* (4 to 20%) was low compared to that in SW+*G. elegans* where 77 % of phosphate was lost by day 2 (Fig. 5).

The biochemical composition of *G. elegans* is presented in Tables 5 and 6. The results show that protein content of *G. elegans* in SBE significantly (p<0.05) increased from 3.50±0.19 to 3.80±0.26% DW after 12 days, while in seawater, it decreased from 3.50±0.19 to 2.67±0.13% DW. The carbohydrate content of the seaweed in SBE significantly (p<0.05) decreased from 51.4±0.3 to 41.4±0.8% DW and that in seawater to 50.6±0.6% DW after 12 days. Ash content was not significantly (p>0.05) different during the cultivation period.

The pigment content including chlorophyll *a*, carotenoids and phycobiliproteins (p>0.05) was higher in *G. elegans* grown in SBE than in SW (Table 5).

The maximum quantum yield (F_v/F_m) of *G. elegans* in SBE increased from 0.60±0.02 to 0.67±0.01 after 12 days. The maximum quantum yield of *G. elegans* showed healthy condition (>0.5) without any indication of stress especially in the SBE+*G. elegans* treatment at the end of the experiment on day 12. Photosynthetic efficiency (α) in *G. elegans* in SBE ranged from 0.41-0.60 which increased with chlorophyll *a* and carotenoids content after 12 days.

The average agar yield in *G. elegans* grown in SBE (25.90±1.36 % DW) was significantly (p<0.05) higher than that in the seawater (21.20±0.73% DW) treatment (p<0.05) on day 12 (Table 6). Gel strength of agar from *G. elegans* grown in SBE (654.3 ±16.5 g cm^{-2}) was significantly (p<0.05) higher than samples cultivated in seawater (604.4±32.0 gcm^{-2}). Agar gelling temperature of the *G. elegans* grown in SBE (36.0±0.4^oC) was significantly (p<0.05) lower than agar of seaweeds grown in seawater (45.2±1.7^oC). No significant difference (p>0.05) was observed for melting temperature of agar from seaweeds grown in SBE (84.4±1.6^oC) and that grown in seawater (83.2 ±1.2 ^oC) (Table 6).

Table 5: Biomass and biochemical composition of *Gelidium elegans* in different treatments on day 12 of culture.

Biochemical composition	Treatment		*T*-ratio & *P*
	SBE+ *G. elegans*	SW+ *G. elegans*	
Biomass[a] (g FW per tank)	532.00 ±21.00	453.00 ±5.00	3.20*
Protein (% DW)	3.80 ±0.26	2.67 ±0.13	6.85*
Carbohydrate (% DW)	41.40 ±0.8	50.60 ±0.6	-14.58*
Agar yield (% DW)	25.90 ±1.40	21.20 ±0.70	9.00*
Ash (% DW)	10.70 ±0.39	11.20 ±0.36	-1.90^{ns}
Chlorophyll *a* (mg g^{-1})	0.11 ±0.01	0.09 ±0.01	1.85^{ns}
Carotenoid (µg g^{-1})	43.20 ±4.98	41.40 ±1.87	5.62^{ns}
Phycobiliprotein (µg g^{-1})	0.92 ±0.29	0.58 ±0.04	2.00^{ns}

*= significant difference at $p<0.05$; ns=not significant; each value represents a mean±SD (n=9); a=(n=3); SBE+*G. elegans*= Shrimp broodstock effluent and *G. elegans*; SW+*G. elegans*= Seawater and *G. elegans*; T=independent sample t-test

Table 6: Physico-chemical properties of agar extracted from *Gelidium elegans.*

Agar properties	df	Treatment		*T*-ratio & *P*
		SBE+*G. elegans*	SW+*G. elegans*	
Agar yield (% DW)	16	25.9 ±1.4	21.2 ±0.7	9.0*
Melting temperature (^oC)	16	84.4 ±1.6	83.2 ±1.2	1.84^{ns}
Gelling temperature (^oC)	16	36.0 ±4.0	45.2 ±1.7	-6.32*
Gel strength (g cm^{-2})	16	654.3 ±16.5	604.4 ±32.0	4.15*

* =significant difference at $p<0.05$; ns=not significant; Values presented as mean ±SD (n=9). SBE+*G. elegans*=*G. elegans* grown in shrimp broodstock effluent; SW+*G. elegans*=*G. elegans* grown in seawater; *T* =independent sample t-test

Discussion

In the present study, *G. elegans* was shown to grow in SBE, with high uptake affinity for nutrients and biomass production. *Gelidium* species are perennial, small and have low growth rates (Friedlander, 2008), where daily growth rate is dependent on species, growth conditions and culture duration (Marinho-Soriano *et al*., 2009a). The RGR of *Gelidium* in the laboratory has been reported for *G. sesquipedale* (1.9%), *Gelidium* sp. (3.8%) and in *in situ* studies for *G. amansii* (6.7%), *G. sesquipedale* (3%), *G. robustum* (1.0-3.6%) and *Gelidium* sp. (13.2%) (Friedlander, 2008). The highest RGR was reported for *G. rex* (33.3%) (Rojas *et al*., 1996), while a RGR of 21.0% for *G. robustum* was obtained in short-term laboratory experiments with 150µM of phosphate supplementation. In open sea conditions, *G. robustum* had a RGR of 1.0% (Pacheco-Ruiz and Zertuche-Gonzalez, 1995). The mean RGR (1.40±0.34% d^{-1}) of *G. elegans* in SBE tanks were lower than the previously reported values. The low growth rate of *G. elegans* in this study may be attributed to the fact that it was transplanted from Korea where the ambient environment is different from the Malaysian environment. *Gelidium* usually grows best at 15-20 °C, but is

able to tolerate higher temperatures (McHugh, 2003).

The SBE with a C:N:P ratio of 46:14:1 was not limiting in general (Phang and Ong, 1988). In the present study, the seaweed was found to be able to uptake nutrients from SBE and produced a higher growth rate (1.40% d^{-1}) and biomass production (3.55 g L^{-1} FW; 18.2% increase in weight) compared to that in seawater (control) (0.16% d^{-1}; 3.02 gL^{-1} FW). The positive effect of salmon effluent on production of *G. chilensis* in tank culture was reported from Chile (Buschmann *et al*., 1996; Zhou *et al*., 2006). *Gelidium* spp. can adapt to low or high salinity and also survive in low nutrient conditions (McHugh, 2003). In the present study, the low growth rate of *G. elegans* grown in seawater can be attributed to nutrient limitation. The lower content of chlorophyll *a* in *G. elegans* grown in seawater tanks can be another reason for low growth rates. A study on *G. pulchellum* (Sousa-Pinto *et al*., 1996) showed maximum growth rate (10 % d^{-1}) under continuous irradiance of 130–240 μmol photons m^{-2} s^{-1} and aeration. In tropical waters enhanced nitrogen and phosphorus levels and water movement can prevent seaweed bleaching by reducing effects of high light and temperature (Santelices, 1987). This is probably why the *G. elegans* pigment content in our study reduced in seawater compared to the SBE treatment. In addition, the SBE provided more nutrients with every water change. Fertilization of *Gelidium* spp. with ammonium or nitrate increases pigment concentration and promotes growth if the additions are made in the presence of phosphorus (Santelices, 1987).

Seaweeds have been proposed as good biofilters in the uptake of nitrogen sources (NH_3-N, NO_3-N and NO_2-N) from aquaculture effluents (Marinho-Soriano *et al*., 2009). Few studies focused on phosphate uptake (Kang *et al*., 2011). Red algae are considered efficient in nutrient uptake due to their abilities to store great reserves of nutrients (Marinho-Soriano *et al*., 2009). In the present study, we concurrently evaluated the biofiltering efficiencies of *G. elegans* for nitrogen and phosphate sources. NH_3-N was removed at the highest rate (>97%) from SBE + *G. elegans* tanks, showing that *G. elegans* took up the NH_3-N for metabolism and storage. NO_2-N uptake was the next highest (79 %) followed by NO_3-N (48 %) and then PO_4-P (20 %) during 12 day culture (Figs. 2–5). Nutrient reduction in control tanks (SBE and SW) may be due to uptake of nutrients by microphytes especially cyanobacteria (Mwandya *et al*., 2001). However, since all tanks were provided with continuous aeration, some of the ammoniacal-nitrogen may have been lost from physical processes.

In this study, *G. elegans* was effective as a biofilter. *G. elegans* exhibited a higher affinity to uptake NH_3-N especially in SBE tanks compared to NO_2-N and NO_3-N. The *G. elegans* in SBE exhibited the maximum percentage of removal efficiency for NH_3-N on days 2 (86 %),

8 (89 %) and 12 (100 %). These results were similar to those previously reported in *Ulva* sp. and *Gracilaria* sp., which exhibited higher NH_4^+ uptake than NO_3 and NO_2 (Kang *et al*., 2011). Similar results were obtained for *Gracilaria vermiculophylla, G. caudata* and other species of *Gracilaria* (Jones *et al*., 2001; Marinho-Soriano *et al*., 2009; Abreu *et al*., 2011). Ammonium is the preferred nitrogen source due to lower energy requirement for assimilation (Lobban and Harrison, 1997), and the low levels ($\leq$0.05 mg L^{-1}) were not inhibitory (Abreu *et al*., 2011) to NO_3-N uptake by the *Gelidium* species. NH_4^+ can be directly incorporated into proteins, amino acids and pigments (Shpigel and Neori 2007; Kim *et al*., 2007; Figueroa *et al*., 2009), and being the most reduced form of inorganic nitrogen, it is from the energetic point of view the most useful nitrogen source for seaweed growth (Marinho-Soriano *et al*., 2009). However some species such as *Laminaria groenlandica* can simultaneously assimilate NH_3-N, NO_3-N and NO_2-N (Harisson *et al*., 1986).

Some studies have shown that seaweeds such as *Gracilaria chilensis, G. lemaneiformis, G. edulis, Ulva lactuca and U. rotundata,* can assimilate about 90 % of the ammonium in fish culture (Buschmann *et al*., 1996; Jones *et al*., 2001; Zhou *et al*., 2006) and 70.5 % for *Kappaphycus alvarezii* cultured with milkfish (*Chanos chanos*) (Hayashi *et al*., 2008). *Gracilaria arcuata* and *Ulva lactuca* were shown to have removal efficiencies of 66.47 – 86.92% and 58.13 – 84.74% for total ammonia nitrogen, respectively, in an integrated study on fish and seaweeds (Al-Hafedh *et al*., 2012). In general, seaweeds growing in nitrogen saturated conditions show luxury uptake for nitrogen sources and store them for growth during periods of nitrogen starvation (Abreu *et al*., 2011).

The maximum removal efficiencies of *G. elegans* in SBE for nitrate (48 %) and phosphate (20 %) in our experiments were similar with the values of 49.6 % (NO_3-N) and 12.3 % (PO_4-P) that was reported by Marinho-Soriano *et al*., (2009) with *Gracilaria caudata*, and higher than removal efficiencies of *Eucheuma* sp. from a farm in the Philippines, where reductions of 24 % in nitrate and nitrite concentrations and 6 % in phosphate concentration, were obtained (Doty, 1987). In a recirculating system where the *Penaeus latisulcatus* was co-cultured with *Ulva lactuca*, the total ammonia nitrogen and phosphate removals were 59 – 81% and 50-55% respectively, with no adverse effects on the growth and survival of the shrimp (Khoi and Fotedar, 2011).

Studies have shown that phosphate can be taken up efficiently in integrated fish-*Ulva* or *Gracilaria* cultivation systems. Phosphate uptake for *Gracilariopsis chorda* (38.1%), *Ulva pertusa* (30.6%) and *Saccharina japonica* (20.2%) were reported (Kang *et al*., 2011). Hayashi *et al*. (2008)

reported of 26.8 % phosphate removal in an integrated culture of *Kappaphycus alvarezii* with fish. Jones *et al*. (2001) showed that, phosphate concentration was reduced in shrimp effluents from 3.30 to 0.16 µM (95 %) by *Gracilaria edulis* cultivated in tanks. For *G. elegans*, the maximum PO_4-P removal was 20 % in SBE + *G. elegans* (Fig. 5). In general, the SBE produced more *G. elegans* biomass that contained higher protein (3.8%DW), chlorophyll *a* (0.11mg g^{-1}), carotenoid (43.2µg g^{-1}), phycobiliprotein (0.92µg g^{-1}) and agar (25.9%DW) contents than that in seawater (Table 5). The carbohydrate content in *G. elegans* grown in SBE was lower than that in the seawater (control) treatment. The protein content in red seaweeds is reported to range from 0.94 to 31.03 % DW (Dere *et al*., 2003). High protein content in *G. elegans* cultured in SBE showed that the seaweed may have absorbed and stored excess nitrogen in the form of protein. The nitrogen supply to seaweeds affects the protein content (Vergara *et al*., 1995) and the photosynthetic capacity (Pérez-Lloréns *et al*., 1996; Barufi *et al*., 2011). The maximum quantum yield (F_v/F_m) of *G. elegans* ranged from 0.60 to 0.67, showing healthy conditions (>0.5) without any indication of stress especially in SBE+*G. elegans* treatment at the end of the experiment on day 12. Photosynthetic efficiency in *G. elegans* grown in SBE treatment increased with enhancement in both chlorophyll *a* and carotenoids content after 12 days. Similar results were obtained for *Ulva lactuca* co-cultured in 80L tanks with *Haliotis* over a 14 day experiment (Cahill *et al*., 2010).

G. elegans in SBE tanks became darker in colour and more ramified than *Gelidium* from the seawater and from the inoculum, while depigmentation and loss of biomass was observed in the seaweeds cultivated in seawater. Darker colour of thalli in SBE may be due to the high nutrient concentration. In low nitrogen concentration, the thalli of red algae turn to light colour whereas well-fertilized thalli are dark, because they are able to store nitrogen in the red pigment phycoerythrin and can remobilize the nitrogen in the pigment to support growth in case of nitrogen deficiency (Zhou *et al*., 2006). Content of chlorophyll *a* in *Ulva lactuca* grown in high nutrient levels was reported higher than in low levels of nutrient based on tank culture system (Figueroa *et al*., 2009). Nitrogen supply in the culture medium can influence pigment content, protein and carbon uptake in many species of seaweeds (Pinchetti *et al*., 1998; Figueroa *et al*., 2009).

The *Gelidium* species are the best sources of bacteriological agar and agarose in the world (Santelices, 1987; McHugh, 2003). They are able to produce high quality agar with low gelling and high melting temperature (McHugh, 2003). Agar yields vary between 6 % and 71 % in the literature, but 20 % being the common value (Troell *et al*., 1999). In the present study *G. elegans* cultivated in SBE and seawater (control) contained 25.90 and 21.20 % DW agar, respectively. This is

slightly lower than the agar yield in other *Gelidium* spp., that is, 27 % in *G.rex*, 25–35 % in *G. latifolium* , 25–31 % in *G. chilense* and 50 % in *G. pusillum* (Santelices, 1987). The agar content of *Gelidium* varies with species, location, season, extraction methods and environmental conditions during culture (Santelices, 1987). The agar content in *G. amansii* (renamed *G. elegans*, Kim *et al*., 2012) was 25–30% and did not change significantly after acclimatisation in Malaysia and culture in SBE. In this study agar extracted from *G. elegans* cultured in SBE showed high melting temperature (84.4^{o}C) and low gelling temperature (36^{o}C), indicating their commercial importance being within the range of the United States Pharmacopoeia (USP) standards (gelling temperature 32–43^{o}C, melting temperature >85^{o}C) and similar to commercial agar (Meena *et al*., 2008). The low gelling temperature of *G. elegans* in agar gel indicates that it is more useful for bacteriological and biotechnological applications. Gel strength of agar in *G. elegans* cultivated in SBE was significantly higher (654.3 g cm^{-2}) than that cultivated in seawater (604.4 g cm^{-2}), making it a good food-grade agar. Kim *et al*. (2010) showed that *G. amansii* (now renamed *G. elegans*) contained 72 to 80% carbohydrate and 33.7% galactose and on fermentation produced an ethanol yield of 93%. The agar extracted from *G. elegans* may therefore be utilised for bioethanol production as an alternative source of biofuel, providing for a "waste to wealth" strategy in bioremediation.

G. elegans after acclimatization to Malaysian conditions was shown to be able to grow in shrimp broodstock effluent, serving as a biofilter for nutrients especially ammoniacal-nitrogen. It grew well and produced a high value biomass which contains protein, carbohydrate and food-grade agar, pulp, and bioethanol.

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