

Physiological responses of two seaweed biofilter candidates, *Gracilariopsis bailiniae* Zhang et Xia and *Hydropuntia edulis* (S Gmelin), to nutrient source and environmental factors

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Abstract. Two gracilarioid species, *Gracilariopsis bailiniae* and *Hydropuntia edulis*, were compared based on their growth under different temperature and salinity levels and nitrogen source and on their photosynthetic responses under different irradiance levels. Results show that growth of *Gp. bailiniae* was significantly higher than that of *H. edulis*. Both species were euryhaline and had optimum growth rates at 27°C (16.06 % d⁻¹ ± 0.10 for *Gp. bailiniae* and 9.53 % d⁻¹ ± 0.62 for *H. edulis*) under both ammonium and nitrate enrichment. *Gracilariopsis bailiniae* was able to use both N-forms as a nitrogen source in all temperature and salinity levels tested. Meanwhile, the interactive effect of nitrogen source with temperature was observed for *H. edulis* with plants grown in nitrate enrichment showing significantly higher growth rates than those in ammonium. Nitrate enrichment also resulted to higher growth rates for *H. edulis* in all salinity levels tested. Photosynthetic rates of *Gp. bailiniae* were higher than *H. edulis*. We also found a two-fold difference between the maximum photosynthetic rate (P_{max}) of *Gp. bailiniae* (12.41 ± 1.81) and that of *H. edulis* (6.44 ± 0.62). However, photosynthetic efficiency (α) was significantly higher in *H. edulis* than in *Gp. bailiniae* while compensation and saturation point irradiance levels were similar in both species.

Key words: *Gracilaria* spp., growth, photosynthesis, nitrogen source, environmental factors.

Introduction. The genus *Gracilaria* and its related genera are among the most economically-important seaweed species due to their value as human food and as a source of agar. Due to their economic value and inherent capacity to filter and store nitrogen in excess of their immediate requirements, gracilarioids have also been considered as ideal biofilter candidates for integrated multi-trophic aquaculture systems (IMTA) (Lapointe 1985; Troell et al 2003). In IMTA systems, fish aquaculture is integrated with other organisms such as seaweeds which serve as an extractive component for dissolved nutrients in the wastewater. These plant-available nutrients used by the seaweed are then converted into biomass that could generate additional revenue to the integrated system. Among these nutrients, ammonium is considered the most dominant (Neori 1996; Carmona et al 2001).

Seaweed biofiltration studies in the Philippines are relatively recent and have only been done for *Kappaphycus* species (Rodrigueza & Montano 2007). In selecting a good seaweed biofilter, it is important to assess its growth responses and nitrogen preference under different environmental variables in order to be assured of tolerant strains for cultivation. High growth rates, commercial value and a preference for ammonium as the nitrogen source are among the criteria that need to be considered (Neori 1996; Buschmann et al 2001; Carmona et al 2001; Neori et al 2004). In this study, we attempt to investigate two gracilarioids, *Hydropuntia edulis* (S Gmelin) (= *Gracilaria edulis* (S Gmelin)) and *Gracilariopsis bailiniae* Zhang et Xia (= *Gracilaria heteroclada* Zhang et Xia, = *Gracilariopsis bailiniae* Zhang et Xia) as potential biofilters based on their physiological

responses to two nitrogen sources and important environmental factors such as temperature, salinity and irradiance. These two species were selected among the numerous gracilarioids in the Philippines due to their potential affinity for areas with high nutrient concentration. Previous studies on *H. edulis* have demonstrated its suitability as a bioindicator of some heavy metals and nutrient pulses (Jones et al 1996). *Gp. bailinae*, on the other hand, is commonly associated with brackishwater fishponds where it thrives year-round (Luhan 1996; Pondevida & Hurtado-Ponce 1996).

Material and Methods

Sample collection. Samples were obtained in two locations in Iloilo, central Philippines in May 2010 (Figure 1). *Gracilariopsis bailinae* was collected from a shallow brackishwater canal in Leganes, Iloilo while *H. edulis* was collected in an intertidal flat at a depth of 1.5 m in San Dionisio, Iloilo.

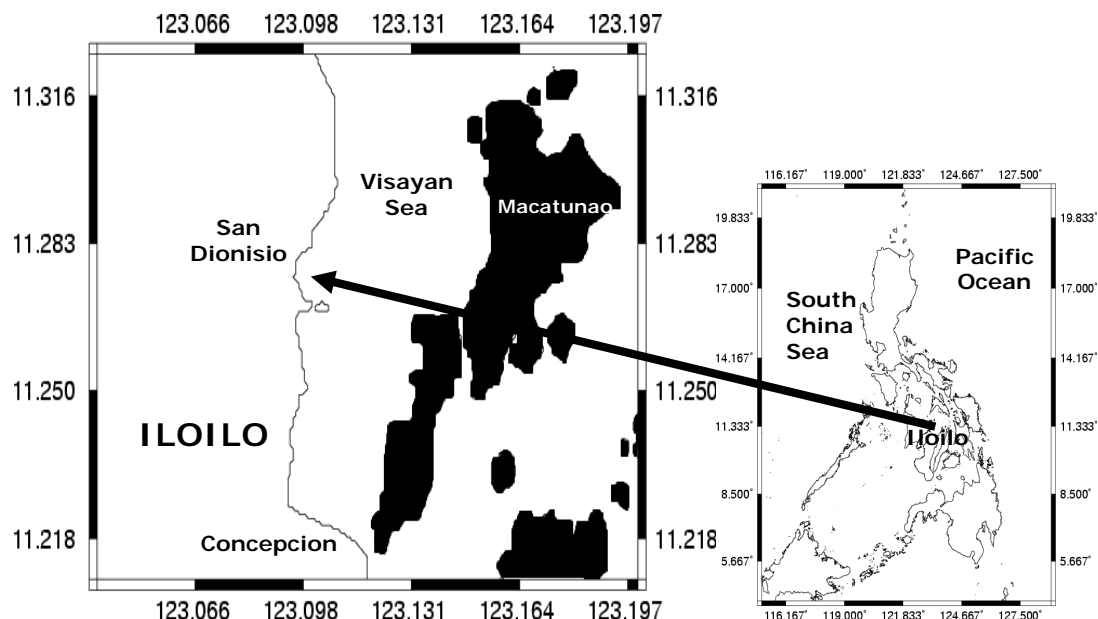


Figure 1. Maps of the Philippines (right) and the coast of the sampling location (left).

Natural substrates in these two areas consisted of mud and rocks for Leganes and San Dionisio, respectively. Each thallus was wrapped in paper towels moistened with seawater and transported to the laboratory in a Styrofoam box. Upon arrival, plants were washed several times with filtered seawater and cleaned using a soft brush to remove debris and epiphytes. Apical tips measuring 2 to 3 cm in length were excised from healthy vegetative sporophytes and placed in a 300-mL flask containing modified Grund medium (McLachlan 1973) prior to the experiment.

Growth experiment. A series of 3 x 4 factorial experiments consisting of 3 temperature (20, 27 and 34 °C) and 4 salinity (18, 25, 32 and 39‰) levels under 2 nitrogen sources (ammonium or nitrate) was conducted in the laboratory for each seaweed species. Apices (0.02-0.03 g) were placed in 300-mL flasks containing 200 mL of modified Grund medium-enriched autoclaved seawater with either ammonium (added as NH₄Cl) or nitrate (added as NaNO₃) as the nitrogen source. Each nitrogen source has a concentration of 0.5 mM and an N:P ratio of 15:1. Different salinity levels were prepared from mixtures of gradually melted frozen seawater and then autoclaved at 120 °C for 60 minutes. Duplicate flasks were used for each temperature-salinity combination. Flasks were placed in a multi-temperature incubator (NK System TG-180) under a 12:12 light:dark photoperiod regime. Growth was monitored weekly for 21 days through wet weight measurements along with the replenishment of the medium. Increments in

growth were expressed as specific growth rate (SGR, % day⁻¹) based on the following formula by Lobban and Harrison (1994):

$$\mu = 100[\ln(N_t/N_o)]/t,$$

where: μ = specific growth rate; N_t = biomass on day t ; N_o = initial biomass; t = time in days.

Photosynthesis experiment. Photosynthetic and respiration responses of *Gp. bailinia* and *H. edulis* were determined at 27°C and irradiance from 50 to 800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Clean and healthy thalli were selected and placed in 300-mL flasks containing autoclaved seawater (35‰) that is replete of nutrients. Triplicate flasks for each species were kept at the desired temperature for 24 hours prior to the analysis to negate possible effects of wound respiration on the oxygen readings. Each flask was incubated in a thermostatic water bath (Eyela NTT-2200; Eyela Bath SB-9, Tokyo Rikakikai Co. Ltd.). Photosynthetic and respiration rates were determined using a Clark-type oxygen electrode (YSI 5100, Yellow Springs, Inc.) fitted inside the flask. All flasks were pre-incubated for 30 minutes in a separate water bath before dissolved oxygen readings were taken. Respiration rate determination was done in flasks entirely covered with foil and the whole set-up covered while photosynthetic rates were measured at photon flux densities from 50 to 800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Irradiance was supplied by a halogen lamp and was standardized using a photometer (LI-COR LI-189). Linear regression equation was fitted to the time-course dissolved oxygen readings obtained and the slope used to calculate dissolved oxygen per dry weight content of the algal material used (in $\text{mg O}_2 \text{ g dry weight}^{-1} \text{ h}^{-1}$). Thalli used were immediately weighed after each analysis and dried in silica gel for dry weight determination.

Photosynthesis-irradiance (P-I) parameters (P_{max} , I_c , I_k , α and R_d) of the seaweed were obtained by fitting light-dependent oxygen evolution data in the following equation described in Platt *et al.* (1980) using KyPlot[®] version 3.0 software:

$$P = P_{max}[1 - \exp(-I/I_k)] \exp(-\beta I/P_{max}),$$

where P is the instantaneous photosynthetic rate per dry weight, P_{max} , the maximum net photosynthesis, I , the photosynthetically-active radiation (PAR), I_k , the saturation point photon fluxes and β , the photoinhibition parameter. At the end of the experiment, tissue samples were dried in silica gel and analyzed for chlorophyll a content using the methodology of Jones *et al* 1994).

Data analyses. Comparisons on the growth and photosynthesis of the two species were made using independent T-test, one-way analysis of variance (ANOVA) and general linear model univariate ANOVA. Differences within data sets were further analyzed using Tukey's test. All statistical analyses were done using SPSS[®] version 16.0.

Results and Discussion

Growth experiment. Significantly higher growth rates were obtained for *Gp. bailinia* compared to *H. edulis* ($F=18.489$; $p<0.005$). Growth of *Gp. bailinia* was mainly influenced by temperature with an optimum growth rate at 27°C ($16.06 \% \text{ d}^{-1} \pm 0.10$) ($F=50.739$; $p<0.005$) (Figure 2). Utilization of both ammonium and nitrate as a nitrogen source by *Gp. bailinia* was evident in all the temperature levels tested. This ability of *Gp. bailinia* to thrive not only in a NO_3^- but also in an NH_4^+ -enriched environment can be particularly useful in brackishwater IMTA systems where this nitrogen form is the most dominant. The high growth rates obtained in this study for *Gp. bailinia* also indicates that high seaweed biomass of commercial value can be supported under such systems. Ammonium also promoted higher degrees of branching in the cultured apices compared to those in nitrate where mainly vertical growth was observed (Figure 3). When cultivating *Gp. bailinia* in the field, higher number of branches are essential for the generation of more apices as these can be developed into new individuals through vegetative growth and then used as seeding stocks in the next growing season.

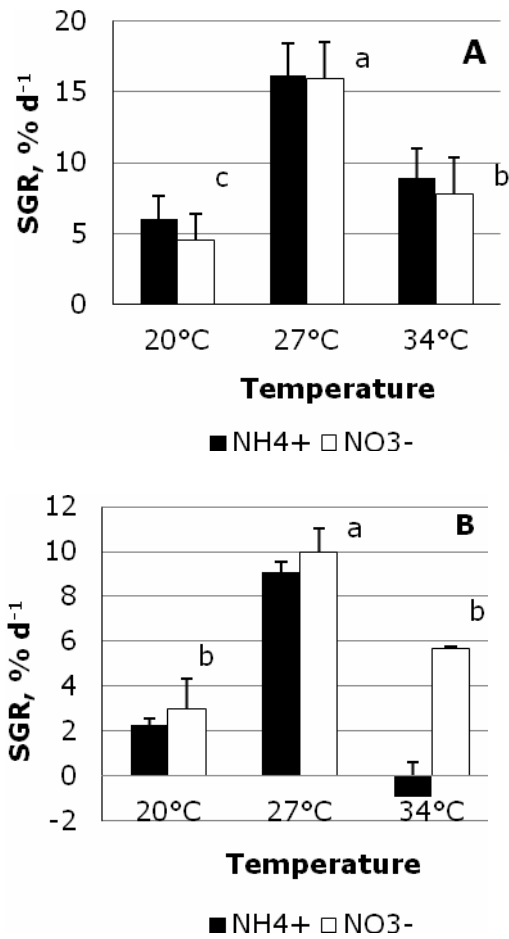


Figure 2. Growth of A) *Gracilariopsis bailinae* and B) *Hydropuntia edulis* under 3 temperature levels and 2 nitrogen sources. Superscripts denote differences at the $p < 0.005$ level among the averaged values at each temperature level.

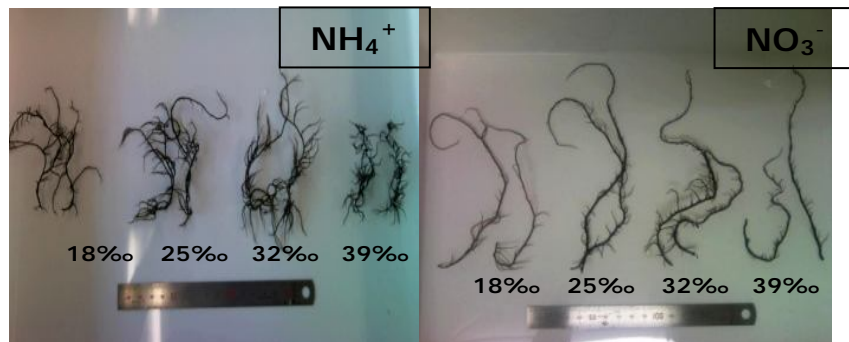


Figure 3. Branching patterns of *Gracilariopsis bailinae* at 27°C under ammonium- and nitrate-enriched medium

For *H. edulis*, growth was found to be a function of both temperature ($F=72.204$; $p < 0.005$) and nitrogen source ($F=24.664$; $p < 0.005$) as well as of their interaction ($F=12.454$; $p < 0.005$). Highest growth rate was also obtained at 27°C ($9.53 \% d^{-1} \pm 0.62$), followed by those at 34°C and 20°C. At 34°C, loss of tissue starting from the 2nd

week of culture was found in apices grown in NH_4^+ while those in NO_3^- grew at an average of $5.70\% \text{ d}^{-1} \pm 0.06$. These results indicate better assimilation of NO_3^- as a nitrogen source for growth of *H. edulis* compared to NH_4^+ . Nitrogen preference has been shown to vary significantly among different species of seaweed (Naldi & Wheeler 1999). Among gracilarioids, higher preference for NH_4^+ than NO_3^- have been reported in nitrogen uptake experiments on *G. foliifera* (D'Elia & DeBoer 1978), *G. pacifica* (Naldi & Wheeler 2002), *G. bursa-pastoris* (Carton et al 2010) and *G. tikvahiae* (Hanisak 1990) while a dual preference for both NH_4^+ and NO_3^- have been shown by *G. cornea* (Navarro-Angulo & Robledo 1999), *G. edulis* (Jones et al 1996) and *Gp. bailinia* (this study). Jones et al (1996) have shown that pigments, total tissue nitrogen and amino acids accumulated in *H. edulis* grown in NH_4^+ and not in NO_3^- and a linear relationship with total tissue nitrogen at 0 to 80 μM of NH_4^+ . At a substrate concentration of 0 to 800 μM NH_4^+ , however, saturation of total tissue nitrogen was observed. High concentration of nutrients in the culture medium has been reported to cause toxicity to the seaweed tissue and result to a decrease in growth rates (Lapointe & Ryther 1979). In this study, a weekly supply of 500 μM NH_4^+ could already be beyond the level of saturation for *H. edulis* and this led to the lower growth rates obtained. Values for NH_4^+ in adjacent coastal waters in San Dionisio, Iloilo have been reported not to exceed 25 $\mu\text{mol L}^{-1}$ without any seasonal pattern (Pondevida & Hurtado-Ponce 1996). High temperature could also have helped in aggravating the physiological stress from ammonium toxicity as shown by tissue loss in *H. edulis* at 34 °C.

Optimum temperature (27 °C) for growth of both *Gp. bailinia* and *H. edulis* was comparable to studies conducted on other tropical southeast asian gracilarioids grown under controlled conditions (Chirapart & Ohno 1993). Both species are well-acclimated to this temperature as it is within the *in situ* values (25 to 32 °C) reported for coastal areas in Iloilo (Luhan 1996; Pondevida & Hurtado-Ponce 1996). In our present study, this temperature supported growth rate (16.06 % d^{-1}) that is the highest reported not only for *Gp. bailinia* but also for other tropical gracilarioids cultured in the laboratory. Rabanal et al (1997) reported maximum growth of *Gp. bailinia* carposporelings in the laboratory at 10.31% d^{-1} . Other culture studies on seaweed species in the same region such as *G. blodgettii* carposporelings showed maximum growth rates at 13.1 % d^{-1} (Carton & Notoya 2008).

No significant differences were found on the growth rates of *Gp. bailinia* in all salinity levels ($F=1.156$; $p>0.05$) and on the nitrogen sources tested ($F=0.0297$; $p>0.05$) (Figure 4). Growth of *H. edulis* was also found to be independent of salinity. However, higher growth rates was obtained for *H. edulis* grown under nitrate enrichment compared to those in ammonium ($F=4.751$, $p<0.05$). Temperature and salinity interaction was not observed for both species ($F=0.195$, $p>0.05$ for *Gp. bailinia* and $F=1.249$, $p>0.05$ for *H. edulis*). Previous studies on *Gp. bailinia* along the Panay coast have shown that salinity did not have an effect on its monthly biomass (Pondevida & Hurtado-Ponce 1996). The euryhaline, apart from the eurythermal characteristic and capacity to utilize both NH_4^+ and NO_3^- for growth, suggests that *Gp. bailinia* has a broader tolerance to different environmental factors. Hence, its ecological success can be assured more than *H. edulis*.

Photosynthesis experiment. Light-dependent photosynthesis of the two seaweed species showed significantly higher photosynthetic rates for *Gp. bailinia* compared to *H. edulis* (Figure 5). Average oxygen production obtained for *Gp. bailinia* and *H. edulis* ranged -0.43 to 14.15 $\text{mg O}_2 \text{ g dry wt}^{-1} \text{ h}^{-1}$ and -0.82 to 6.51 $\text{mg O}_2 \text{ g dry wt}^{-1} \text{ h}^{-1}$, respectively. Table 1 shows the photosynthesis-irradiance parameters obtained from this experiment. We found a two-fold difference between the maximum net photosynthetic rate (P_{max}) of *Gp. bailinia* ($12.41 \pm 1.81 \text{mg O}_2 \text{ g dry wt}^{-1} \text{ h}^{-1}$) and that of *H. edulis* ($6.44 \pm 1.81 \text{mg O}_2 \text{ g dry wt}^{-1} \text{ h}^{-1}$) ($F=32.448$; $p<0.05$) and this can be attributed for the significant difference between the growth rates of the two species. A comparison between *G. foliifera* and *Ulva* sp. showed higher growth rates in the latter that were reflected in its higher photosynthetic capacity (Rosenberg & Ramus 1982). This difference in their photosynthetic capacities particularly on the P_{max} values was associated more to differences in surface area:volume (SA:V) ratio rather than internal thallus and

chloroplast anatomy, among others (Ramus 1978; Littler 1980). While thallus forms of both species used in our study are not that different from each other unlike *Ulva* sp. and *G. foliifera*, branches of *Gp. bailinia*e appear finer than those of *H. edulis*. Branches of *H. edulis* which are cartilaginous generally range from 1.5 to 2.2 mm in diameter while that of *Gp. bailinia*e range from 0.5 to 2.0 (Trono 1997; Terada et al 2000). Littler (1980) found that finely-branched forms of seaweed were more productive than their coarsely-branched counterparts. Such minor morphological difference between the two species could help explain the variations in their photosynthetic capacities and their subsequent effects on growth.

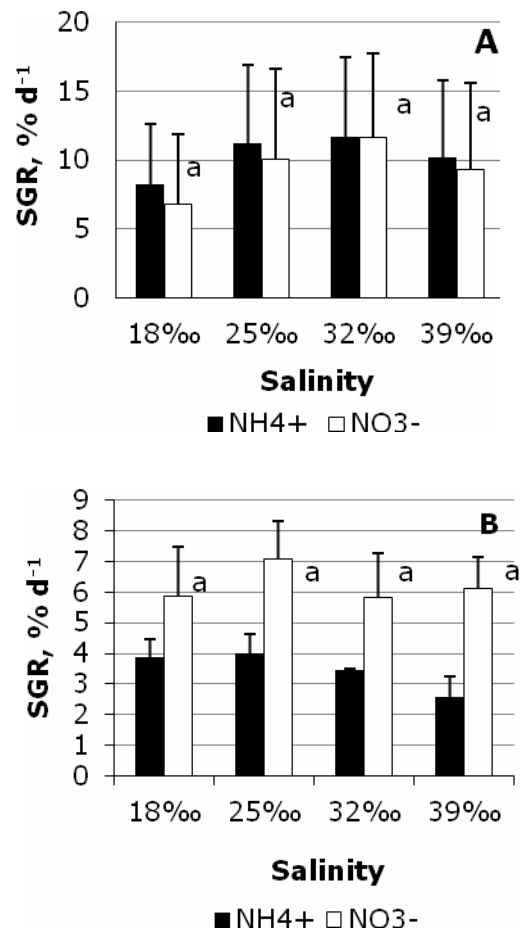


Figure 4. Growth of A) *Gracilariopsis bailinia* and B) *Hydropuntia edulis* under 4 salinity levels and 2 nitrogen sources. Superscripts denote differences at the $p < 0.005$ level among the averaged values of the 4 salinity levels.

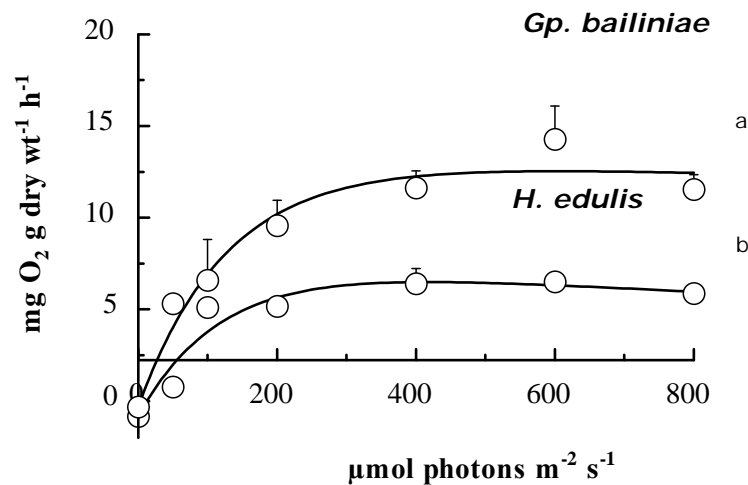


Figure 5. Oxygen evolution rates of *Gracilariopsis bailiniae* and *Hydropuntia edulis* at 27°C and irradiance levels from 0-800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Values with different superscripts are significantly different at the $p < 0.005$ level.

Table 1
Photosynthetic-irradiance (P-I) parameters of *Gracilariopsis bailiniae* and *Hydropuntia edulis* at 27°C and irradiance levels from 0-800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Values with different superscripts are significantly different at the $p < 0.005$ level

P-I Parameters	<i>Gp. bailiniae</i>	<i>H. edulis</i>
Maximum photosynthetic rate, P_{max}	12.41 ± 1.81^a	$6.44 \pm .62^b$
Compensation point irradiance level, I_c	46.22 ± 0.59^a	23.35 ± 9.45^a
Saturation point irradiance level, I_k	123.91 ± 10.02^a	191.74 ± 41.13^a
Photosynthetic efficiency, α	0.11 ± 0.01^a	0.03 ± 0.00^b
Respiration rate, R_d	0.43 ± 0.04^b	0.93 ± 0.43^b

Photosynthetic efficiency (α) was significantly higher in *H. edulis* than in *Gp. bailiniae* ($F=128.000$; $p < 0.005$). Compensation (I_c) and saturation (I_k) point irradiance levels as well as dark respiration rates (R) did not vary between the two species. Saturation point irradiance levels of the two species suggest acclimation to high light levels as these were beyond the values (90 to 112 $\mu\text{mol m}^{-2} \text{s}^{-1}$) typically associated with shade-adapted plants (Dawes et al 1999). However, the higher photosynthetic efficiency obtained from *H. edulis* indicates a better capacity to adapt in low-light level conditions than *Gp. bailiniae*. These responses to irradiance generally translate to the need of these two seaweed species to be cultivated in shallow water in order to maximize their productivity. Chlorophyll *a* levels from the tissue samples used were also not significantly different. Average chlorophyll *a* values were at 0.552 ± 0.06 and $0.604 \pm 0.03 \text{ mg g dry wt}^{-1}$ for *Gp. bailiniae* and *H. edulis*, respectively. This lack of difference between these values shows that photosynthetic capacity was also independent of chlorophyll *a* as a main photosynthetic pigment.

Conclusions. This study shows that *Gp. bailiniae* can be expected to perform better as an IMTA biofilter candidate than *H. edulis* due to its capacity to utilize both NH_4^+ and NO_3^- in generating economically-viable biomass even under extreme temperature and salinity levels. Since growth of *H. edulis* in ammonium enrichment is limited at high temperature levels, only indoor culture systems under controlled temperature may be feasible. Both species require high saturating irradiance levels so cultivation in shallow or well-lighted culture systems is recommended.

Acknowledgements. This study was supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology (Japan-MEXT) research scholarship. We would like to thank the Brackishwater Aquaculture Center of the UP Visayas-CFOS-IA for providing the facilities needed for the maintenance of the seaweed samples.

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Received: 04 October 2011. Accepted: 02 November 2011. Published online: 10 November 2011.

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How to cite this article:

Carton R. J., Caipang C. M., Notoya M., Fujita D., 2011 Physiological responses of two seaweed biofilter candidates, *Gracilariopsis bailinae* Zhang et Xia and *Hydropuntia edulis* (S Gmelin), to nutrient source and environmental factors. *AAAL Bioflux* **4**(5):635-643.