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Effect of salinity and pH on growth and agar yield of *Gracilaria tenuistipitata* var. *liui* in laboratory and outdoor cultivation

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Abstract

Acclimation responses of the red alga *Gracilaria tenuistipitata* var. *liui* collected on the northwest coast of Philippines were determined in laboratory setups and outdoor cultivation tanks in Haifa, Israel. Growth under laboratory conditions was influenced by all three variables studied, namely, temperature (20 or 30 °C), salinity (20, 30 or 39‰) and seawater pH (6.5, 7.0, 8.0 or $\geq$ 9.0). In 250 mL flasks lacking pH control growth was influenced by temperature only at 20 ‰, whereas at 39 ‰, growth rates were similar at 20 or 30 °C. In 500 mL cylinders in which pH was controlled, growth rates were significantly different at a pH of 6.5 and 7.0 for all salinities, with maximal rates occurring in 39 ‰. At pH 8.0, and above, growth rates between salinities were similar and reduced to approximately 50% at a pH of 9.0 compared to rates at a pH of 6.5. Photosynthesis responses generally resembled growth responses both, in 250 mL and 500 mL cultures. In 40-L outdoor tanks, weekly growth and agar yields were apparently enhanced by increasing light intensities (up to full sunlight) and nutrient concentrations (up to 0.2 mM PO_3^{2-} and 2.0 mM NH_4^+), and rates averaged four times higher than rates determined in the smaller flask cultures. This study shows broad salinity tolerance of *G. tenuistipitata* var. *liui* and its ability to sustain growth rates that are among the highest measured for *Gracilaria* spp. in outdoor cultures.

Introduction

The seaweed experimental unit at the Israel Oceanographic & Limnological Research (IOLR) Institute, Israel, has developed an inland seaweed cultivation system for a variety of macroalgae, particularly strains of *Gracilaria*. An advantage of the system is the practical regulation of environmental factors to insure high biomass yields on a yearly basis (Friedlander & Levy, 1995). One important step involves screening potentially valuable seaweeds for their growth characteristics in the laboratory before transfer to outdoor experimental settings. Recently, the growth performance of the subtropical red alga *Gracilaria tenuistipitata* var. *liui* has been studied at the IOLR. This species thrives in the western Pacific and S-E Asia region and one variety, *G. tenuistipitata* var. *liui*, is commonly used as a sea vegetable in the Philippines and cultivated in ponds in Taiwan and China for agar (Haglund & Pedersen, 1993; Tseng & Xia, 1999).

While effects of individual abiotic factors such as light, temperature and salinity on growth and agar properties have been extensively studied in *Gracilaria* species, few, however, examine physiological responses to a combination of factors (Dawes, 1998). For example, estuarine species like *G. tikvahiae* and *G. verrucosa* exhibit broad tolerances to abiotic factors (Lapointe et al., 1984) while oceanic species such as *G. cornea*, are susceptible to minor fluctuations of temperature and salinity (Dawes et al., 1999). Limited reports on *G. tenuistipitata* suggest its broad salinity tolerance (7 ‰ to 47 ‰) and temperature as shown by

Table 1. Concentrations of photosynthetic pigments, chlorophyll *a* (Chl *a*), phycoerythrin (PE) and phycocyanin (PC), and total soluble protein (TSP) for *Gracilaria tenuistipitata* var. *liui* grown in 250-mL flasks at various salinities and temperatures. Data are the average of $n = 3$ biological replicates $\pm$ S.D

Growth Temp (°C)	Growth Salinity (‰)	Chl *a* (mg g^{-1} FW)	PE	PC	TSP (% DW)
20	20	0.34 ± 0.04	0.19 ± 0.03	0.25 ± 0.04	7.5 ± 2.5
	30	0.53 ± 0.03	0.36 ± 0.03	0.31 ± 0.10	22.5 ± 5.4
	39	0.51 ± 0.12	0.47 ± 0.07	0.32 ± 0.05	27.5 ± 2.8
30	20	0.46 ± 0.05	0.19 ± 0.01	0.25 ± 0.02	20.0 ± 5.0
	30	0.44 ± 0.07	0.27 ± 0.04	0.25 ± 0.03	22.5 ± 2.5
	39	0.67 ± 0.14	0.51 ± 0.09	0.32 ± 0.06	27.5 ± 7.4

its photosynthesis and growth (Chiang, 1981; Haglund & Pedersen, 1993). Another important factor affecting growth of seaweed cultures is the high seawater pH resulting from photosynthetic uptake of inorganic carbon (Maberly, 1990; Beer, 1994). Lower concentrations of HCO_3^- and CO_2 at high pH and low diffusion rates of CO_2 in seawater (Johnson, 1982), may in several occasions reduce rates of CO_2 uptake, photosynthesis and algal growth (Beer & Israel, 1990; Beer, 1994).

The aim of this study was to assess the influence of salinity and pH on the growth responses of *Gracilaria tenuistipitata* var. *liui*, particularly its nutrient and light requirements. The long-term goal is to define optimal conditions for maximal yields in the intensive growth system established at the IOLR.

Materials and methods

Vegetative sporophytes of *Gracilaria tenuistipitata* var. *liui* were collected in Sorsogon south of Luzon, Philippines, and brought to the Israel Oceanographic & Limnological Research (IOLR) Institute in May 1997. They were kept in sterilized, nutrient enriched (Provasoli Enrichment Medium and metal mix PII, PEM-PII; Provasoli, 1968) seawater and continuous aeration at 25 °C. The medium was replaced weekly with fresh seawater and epiphytes or contaminants were gently brushed away.

Approximately 0.5 g fresh weight (FW) alga was placed in 250-mL flasks filled with sterilized seawater (39 ‰ salinity), or seawater diluted to 30 ‰ or

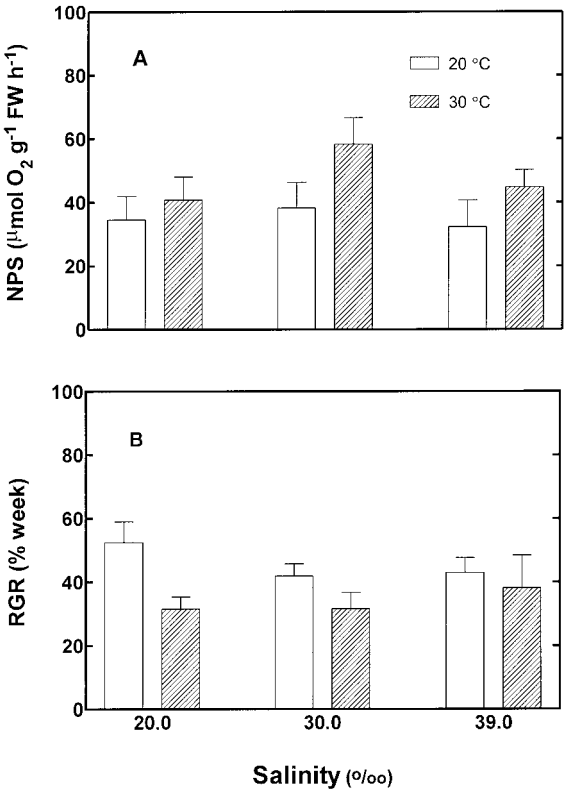

Figure 1. Rates of (A) net photosynthesis (NPS, $n = 3 - 4 \pm$ S.D.) and (B) relative growth (RGR, $n = 4 \pm$ S.D.) for *Gracilaria tenuistipitata* var. *liui* grown at 20 °C or 30 °C in 250-mL flasks using seawater (39 ‰) or diluted seawater solutions (20 ‰ and 30 ‰) and 100 μmol photon m^{-2} s^{-1}.

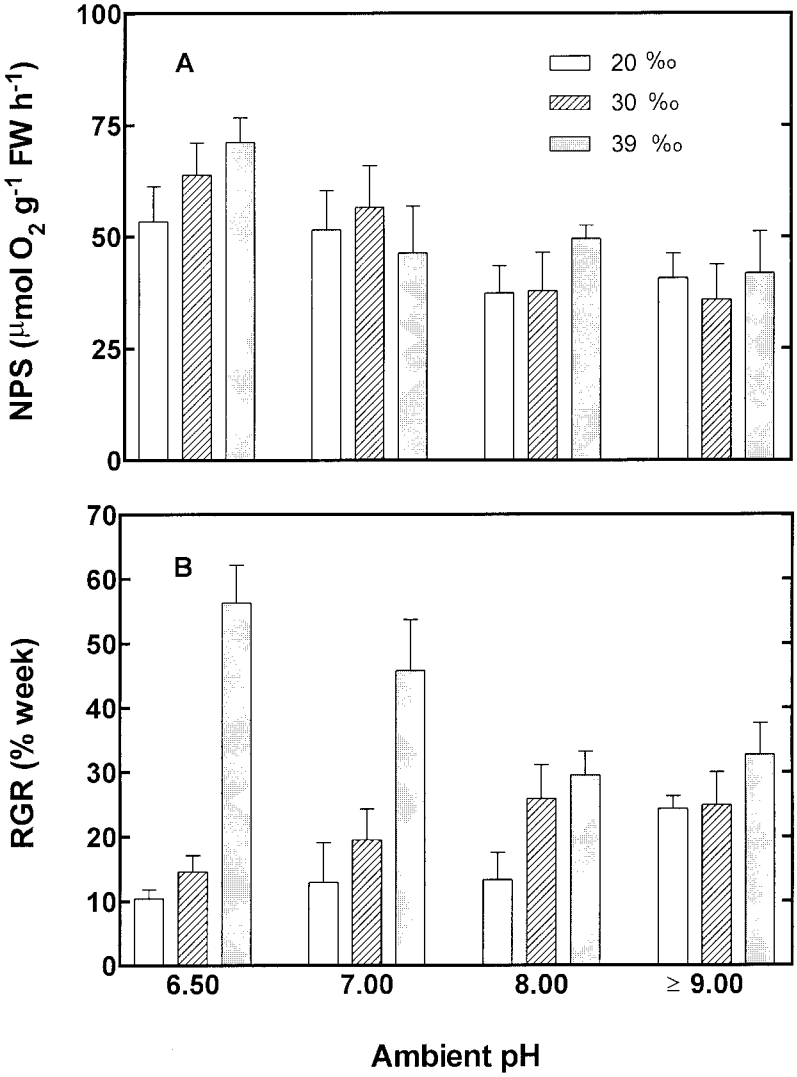

Figure 2. Rates of (A) net photosynthesis (NPS, $n = 3 - 6 \pm$ S.D.) and (B) relative growth (RGR, $n = 3 \pm$ S.D.) for *G. tenuistipitata* grown under ambient pH and salinity gradients in 500-mL glass cylinders at 20 °C and 100 μmol photon m^{-2} s^{-1}.

20 ‰ salinity. Growth solutions were continuously aerated, enriched with PEM-PII and replaced twice a week. The alga was grown in growth chambers at 30 or 20 °C equipped with cool white fluorescent bulbs yielding 100 μmol photon m^{-2} s^{-1} photosynthetic photon flux (PPF) under 12-h photoperiods. Growth experiments consisted of a week of acclimation followed by 4 weeks of measurements. Growth rate was determined using weekly FW increments.

About 1.5 g FW *Gracilaria tenuistipitata* segments were grown in each of eight, 500 mL, glass cylinders equipped with water jackets to keep temperature at 20 °C with a water bath. The cylinders were filled with PEM-PII enriched seawater (39 ‰) or diluted seawater (30 ‰ and 20 ‰) and illuminated 12 h per day at 100 μmol photon m^{-2} s^{-1}. The cylinders were covered with rubber stoppers through which air hoses, CO_2 inlets and pH electrodes (Hanna Instruments, Tel Aviv, Israel) were inserted. The pH electrodes were connected to a multi-channel pH controller which pumped CO_2 into the cylinders to maintain predetermined pH values of 6.5, 7.0 or 8.0. Two cylinders lacking a pH-stat were used as control. The relative concentrations of CO_2 and HCO_3^- were calculated for each salinity addressing the effects of pH, temperature and ionic strength on Ci levels (Beer & Eshel, 1983).

Table 2. Concentrations of HCO_3^- and CO_2 and their ratios as affected by salinity and ambient pH. Calculations were done using PROGRAM CARBON (Beer & Eshel, 1983) and assumed a closed seawater system containing 2.2 mM total inorganic carbon at 20 °C

Salinity ‰	Ambient pH	CO_2 (μM)	HCO_3^- (mM)	HCO_3^-/CO_2
20	6.5	640.2	1.56	2.5
	7.0	252.1	1.94	7.7
	8.0	11.7	1.99	170
	9.0	2.0	1.54	772
30	6.5	578.8	1.62	2.8
	7.0	222.4	1.97	8.8
	8.0	14.3	1.99	139
	9.0	1.6	1.38	861
39	6.5	534.5	1.66	3.1
	7.0	201.8	1.98	9.8
	8.0	14.3	1.93	135
	9.0	1.3	1.27	974

These calculations were done assuming that (1) the total Ci concentration in seawater was 2.2 mM and (2) gas exchange with the atmosphere was minimal and, therefore, the cylinders were regarded as 'closed systems'. The alga was allowed to grow for two consecutive weeks at each salinity and pH range, and growth rates were determined as daily FW increments.

Rates of net photosynthesis were determined from 0.2–0.3 g FW algal segments using a liquid phase O_2 electrode system (Hansatech Ltd, King's Lynn, Norfolk, UK) at 20 °C. The 2.5-mL sample chamber was filled with 0.2-μm filtered seawater, diluted seawater or artificial seawater solutions (ASW, containing 450 mM NaCl, 30 mM $MgSO_4$, 10 mM KCl and 10 mM $CaCl_2$). A flat-tip pH electrode (Hanna Instruments, Tel Aviv, Israel) was inserted in the sample chamber to follow pH shifts during photosynthesis. The alga was illuminated at 150 μmol photon m^{-2} s^{-1} until rates of O_2 release were stable over 10-min periods.

Approximately 100 g FW alga were transferred to 40-L fiberglass tanks supplied with running seawater and continuous aeration at different light levels and nutrient concentrations (Friedlander, 1992). Illumination levels of 20, 50, 70 and 100% sunlight (averaging 1650 μmol photon m^{-2} s^{-1} at the water surface) were created by attaching polypropylene nets on top of the tanks. Once a week the seawater flow was stopped and a mixture of nitrogen and phosphate was supplied for 24-h. Nitrogen was added as NH_4Cl to give final concentrations of 0.1, 0.5 and 2.0 mM and phosphate as NaH_2PO_4 at concentrations one-tenth those of ammonium. Outdoor experiments were carried out for six weeks, including two weeks acclimation at an average seawater temperature of 25.5 ± 1.5 °C.

Chlorophyll *a* was measured from N,N-dimethylformamide extracts according to Moran (1982), and phycobilins, phycoerythrin and phycocyanin, determined from crude extracts according to Moon and Dawes (1976). Total soluble proteins were analyzed spectrophotometrically following 24-h digestion of 20 mg powdered alga (Lowry et al., 1951). Amounts of C and N were measured with a PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, Norwalk, CT, USA) using dried samples. Agar yields and gel strength followed procedures of Craigie and Leight (1978).

The statistical significance of nutrients, PPF and temperature effects and their interactions were analyzed by two-way ANOVA, or tested with Student's t-test (Sokal & Rohlf, 1998).

Results

At 20 °C, *Gracilaria tenuistipitata* showed similar ($p > 0.05$) rates of net photosynthesis (NPS) when grown in 3 salinities using 250 mL flasks (Figure 1A). At 30 °C, the NPS at 30 ‰ averaged 27% higher than NPS at 20 ‰ or 39 ‰ ($p < 0.05$). Relative growth rates (RGR) of *G. tenuistipitata* grown at 20 °C and 20 ‰ were significantly higher ($p < 0.05$) than RGR rates at other salinities and were 40% higher than rates at 30 °C ($p < 0.01$; Figure 1B).

For any particular value of ambient pH, the net photosynthetic rate of *Gracilaria tenuistipitata* grown in 500-mL cylinders at 20 °C was not significantly different between salinities ($p > 0.05$; Figure 2A). In cylinders without pH control the ambient pH increased rapidly to 9.0–9.3 within 2–3 h with the initiation of the light period (data not shown). Relative growth increased significantly ($p < 0.05$) with increasing salinity at pH 6.5 or 7.0, and to a lesser degree at pH 8.0 (Figure 2B).

For any particular salinity, the amounts of major photosynthetic pigments, chlorophyll *a* (chl *a*), phycoerythrin (PE) and phycocyanin (PC) were rather similar at 20 °C and 30 °C. At 39 ‰ chl *a* levels in-

Table 3. Effect of NH_4^+ concentration on relative growth rates (RGR, % week, $n = 12$), C:N ratios ($n = 8$), agar yield (mg 200 g^{-1} FW d^{-1}, $n = 12$) and gel strength (g cm^{-2}, $n = 8$) for *G. tenuistipitata* var. *liui* grown in 40-L tanks. Data are the average of all measurements at 20, 50, 70 and 100% sunlight (1650 μmol photon m^{-2} s^{-1}) during 6 weeks

	NH_4^+ concentration (mM)			
	0	0.1	0.5	2.0
RGR	8.7 ± 6.6^{a}	55.7 ± 13.6^{b}	128.8 ± 38.5^{c}	147.0 ± 52.5^{c}
C:N	44.7 ± 2.8^{d}	46.1 ± 3.8^{d}	17.0 ± 2.7^{e}	10.3 ± 1.9^{e}
Agar Yield	63.4 ± 13.5^{f}	408.3 ± 14.5^{g}	570.9 ± 6.7^{h}	382.9 ± 45.0^{g}
Gel Strength	736.3 ± 14.1^{i}	1109.7 ± 12.9^{j}	1076.6 ± 83.5^{j}	783.5 ± 55.2^{i}

– data sharing similar letters were non-significantly different

creased by 32% and those of PE by 61% as compared to pigment concentrations at 20 ‰. Neither temperature nor salinity affected the amounts of total soluble proteins (TSP) except for plants grown at 20 °C and 22 ‰, which contained approximately 70% lower TSP ($p < 0.01$) than that in other treatments (Table 1). Calculations of CO_2 and HCO_3^- concentrations in the experimental cylinders (regarded as 'closed systems') indicated a strong effect of ambient pH and only a minor effect of salinity on Ci partitioning (Table 2).

Relative growth rates (RGR) of *Gracilaria tenuistipitata* grown in 40-L outdoor tanks increased with increasing concentration of NH_4^+ saturating at 0.5 mM NH_4^+ ($p < 0.05$; Table 3). For a particular NH_4^+ level, increasing PPF had no significant effect on RGR ($p > 0.01$ or 0.05; data not shown). The ratio of C:N increased with decreasing levels of NH_4^+ and was 78% lower for alga grown with 2.0 mM NH_4^+ compared to untreated alga ($p < 0.01$; Table 3).

Daily agar yields of alga grown without NH_4^+ increased 7-fold compared to agar yields from alga grown with 0.5 mM NH_4^+ ($p < 0.05$; Table 3). Gel strength was generally higher in plants grown in 0.1 or 0.5 mM NH_4^+ compared to those grown in 2.0 mM or without NH_4^+ ($p > 0.05$; Table 3). There were no significant effects of increasing PPF level on agar levels, daily agar yields or gel strength ($p > 0.05$; data not shown).

Discussion

Growth experiments suggest that *Gracilaria tenuistipitata* is tolerant to a broad range of salinities with some additional benefit at 20 ‰, in agreement with previous studies (Haglund & Pedersen, 1993). However, decreasing temperatures inhibited the growth of plants in 20 ‰ and 30 ‰ salinities, whereas growth rates were unaffected in regular seawater. In addition, growth was strongly stimulated at 39 ‰ and at a pH of 6.5 and 7.0 possibly due to relatively high CO_2 concentrations at such pH values. Consequently, while algal growth can be made optimal through control of salinity or pH in the laboratory, a cost-effective outdoor cultivation system at 39 ‰ salinities could also support high growth in culture tanks exposed to abundant irradiance and constant mixing through aeration and seawater flow outdoors.

Low water exchange rates in pond or tank cultures can lead to a rapid rise in ambient pH, followed by Ci limitation, particularly at high stocking densities (Friedlander & Levy, 1995; Israel & Friedlander, 1998). Indeed, pH values of 9.0, or above, commonly occur in culture systems and may inhibit growth (Frost-Christensen & Sand-Jensen, 1990). However, high growth rates have been measured in non-aerated pond cultures of *G. tenuistipitata* in which the pH quickly increased to values around 10 (Haglund & Pedersen, 1992). In this study, growth rates of *G. tenuistipitata* were maximal at a pH of ≥ 9.0 in the laboratory and substantial rates were observed in outdoor cultures, similar to *Gelidiopsis* sp. grown under high seawater pH (Israel & Friedlander, 1998). The four times higher growth of *G. tenuistipitata* in outdoor tanks may be due to nutrient and Ci limitations, or water motion in laboratory setups. Growth rates in 40 m^3 outdoor tanks at 17–24 °C and 7 ‰ were only 28% per week (Haglund & Pedersen, 1993) contrasting the much higher growth rates of *G. tenuistipitata* measured in this study (using optimal N and P in 40-L tanks with 39 ‰ seawater).

Higher salinities produced a general increment in the amounts of chl *a*, phycobilins as well as total sol-

uble proteins in contrast to the limited effect of the two temperatures used in culture. Increased agar yields and gel strength correlated with high growth rates for *G. tenuistipitata* in this investigation and for *G. conferta* in an earlier study (Friedlander, 1991). Thus, growth and chemical constituents were affected by concentration of nutrients rather than by PPF levels. This could result from low light saturation point for growth, which seems to occur at about 330 μmol photon m^{-2} s^{-1} (corresponding to 20% of 1,650 μmol photon m^{-2} s^{-1}, the average sunlight during the experimental period).

The high C:N ratios found in *Gracilaria tenuistipitata* at low levels of N and P is expected since low nitrogen levels would limit uptake, intracellular levels and algal growth (Friedlander et al., 1991). Also high C:N ratios in both micro- and macroalgae are correlated with high CO_2 concentrations in the seawater medium (Burkhardt & Riebesell, 1997). However, the magnitude of C:N change caused by CO_2 availability is moderate compared to effects generally observed under severe N or P limitation. Red algae inhabiting nutrient-rich seawater in an upwelling system showed low C:N values of 7–12 (Levitt & Bolton, 1990), similar to *G. tenuistipitata* grown in 40 m^3 tanks with N-rich brackish water (Haglund & Pedersen, 1993).

In conclusion, the present study supports broad salinity tolerance and shows rapid growth of *Gracilaria tenuistipitata* in an experimental tank culture system. Growth rates were among the highest so far reported for this species; about 170% per week under optimal nutrient and irradiance levels (i.e. 0.5 mM NH_4^+ and 0.05 mM PO_4^{2-} at any sunlight above 300 μmol photon m^{-2} s^{-1}). As maximal growth also correlated with high agar yields, *G. tenuistipitata* appears to be a promising seaweed for intensive cultivation in inland culture systems.

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References

Beer S (1994) Mechanisms of inorganic carbon acquisition in marine macroalgae (with special reference to chlorophyta). In Chapman DJ, Round F (eds), Progress in Phycological Research, Biopress Ltd., Bristol, England: 179–207.

Beer S, Eshel A (1983) Photosynthesis of *Ulva* sp. II. Utilization of CO_2 and HCO_3^- when submersed. J. exp. mar. Biol. Ecol. 70: 99–106.

Beer S, Israel A (1990) Photosynthesis of *Ulva lactuca*. IV. pH, carbonicanhydrase, and inorganic carbon conversions in the unstirred layer. Pl. Cell Environ. 13: 555–560.

Burkhardt S, Riebesell U (1997) CO_2 availability affects elemental composition (C:N:P) of the marine diatom *Skeletonema costatum*. Mar. Ecol. Progr. Ser. 155: 67–76.

Chiang YM (1981) Cultivation of *Gracilaria* (Rhodophycophyta, Gigartinales) in Taiwan. In Levring T (ed.), Proceedings of Xth International Seaweed Symposium. Walter de Gruyter & Co., Berlin, New York, 569–574.

Craigie JS, Leigh C (1978) Carrageenans and agars. In Hellebust JA, Craigie JS (eds), Handbook of Phycological Methods. Cambridge University Press. Cambridge, 109–131.

Dawes CJ (1998) Marine Botany. Second edition. J. Wiley & Sons, New York. 480 pp.

Dawes CJ, Orduna-Rojas J, Robledo D (1999) Response of the tropical red seaweed *Gracilaria cornea* to temperature, salinity and irradiance. J. appl. Phycol. 10: 419–425.

Friedlander M (1991) Growth rate, epiphyte biomass and agar yield of *Gracilaria conferta* in an annual outdoor experiment. 1. Irradiance and nitrogen. Biores. Technol. 38: 203–208.

Friedlander M (1992) *Gracilaria conferta* and its epiphytes: the effect of culture conditions on growth. Bot. mar. 35: 423–428.

Friedlander M, Krom MD, Ben-Amotz A (1991) The effect of light and ammonium ongrowth, epiphytes and chemical constituents of *Gracilaria conferta* in outdoor cultures. Bot. mar. 34: 161–166.

Friedlander M, Levy I (1995) Cultivation of *Gracilaria* in outdoor tanks and ponds. J. appl. Phycol. 7: 315–324.

Frost-Christensen H, Sand-Jensen K (1990) Growth rate and carbon affinity of *Ulva lactuca* under controlled levels of carbon, pH and oxygen. Mar. Biol. 104: 497–501.

Haglund K, Pedersen M (1992) Growth of the red alga *Gracilaria tenuistipitata* at high pH. Influence of some environmental factors and correlation to an increased carbonic-anhydrase activity. Bot. mar. 35: 579–587.

Haglund K, Pedersen M (1993) Outdoor pond cultivation of the marine red alga *Gracilaria tenuistipitata* var. *liui* Zhang et Xia (Gracilariales, Rhodophyta) in brackish water in Sweden. Growth, nutrient uptake, cocultivation with rainbow trout and epiphyte control. J. appl. Phycol. 5: 271–284.

Israel A, Friedlander M (1998) Inorganic carbon utilisation and growth abilities in the marine red macroalga *Gelidiopsis* sp. Isr. J. Plant Sci. 46: 117–124.

Johnson KF (1982) Carbon dioxide hydration and dehydration kinetics in seawater. Limnol. Oceanogr. 27: 849–855.

Lapointe BE, Tenore KR, Dawes CJ (1984) Interaction between light and temperature on the physiological ecology of *Gracilaria tikvahiae*. 1. Growth, photosynthesis and respiration. Mar. Biol. 80: 161–170.

Levitt GJ, Bolton JJ (1990) Seasonal primary production of understory Rhodophyta in an upwelling system. J. Phycol. 26: 214–220.

Lowry OM, Rosebrough NT, Farr AL, Randall JR (1951) Protein measurement with folic phenol reagent. J. biol. Chem. 193: 263–275.

Maberly SC (1990) Exogenous sources of inorganic carbon for photosynthesis by marine macroalgae. J. Phycol. 26: 439–449.

Moon RE, Dawes CJ (1976) Pigment changes and photosynthetic rates under selected wavelengths in the growing tips of *Eucheuma isiforme* v. *denudatum* during vegetative growth. Br. phycol. J. 11: 165–175.

Moran R (1982) Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide. Plant Physiol. 69: 1376–1381.

Provasoli L (1968) Media and prospects for cultivation of marine algae. In Watanabe A, Gattori A (eds), Culture and Collection of Algae. Proc. U.S.-Japan Conf., Hakonte, Jap. Soc. Plant Physiol.: 63–75.

Sokal RR, Rohlf FJ (1998) Biometry. The principles and practice of statistics in biological research. Third edition. Freeman WH. New York. 887 pp.

Tseng CK, Xia B-M (1999) On the *Gracilaria* in the Western Pacific and the Southeastern Asia region. Bot. mar. 42: 209–217.