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Temperature- and light-dependent growth and metabolism of the invasive red algae *Gracilaria vermiculophylla* – a comparison with two native macroalgae

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We conducted two temperature experiments to investigate the invasion success of the coarsely branched red algae, *Gracilaria vermiculophylla*. Our working hypothesis was that the coarsely branched *G. vermiculophylla*, with well-known broad environmental tolerances, would have physiological traits in-between the typical *r* and *K* strategies. A factorial experiment provided light-dependent models of growth rate at six temperatures, with maximum growth of 0.045 day^{-1} at 20°C. Light-saturated growth and maximum light utilization efficiency both displayed a bell-shaped temperature dependency, with optima at 21°C and 23°C respectively. The minimum light required to maintain growth was low (<1 µmol photons m^{-2} s^{-1}) for lower temperatures (5–20°C) and increased exponentially to 7 µmol photons m^{-2} s^{-1} at 30°C, documenting that *G. vermiculophylla* has a wide tolerance to low light levels under temperature ranges occurring in the upper littoral zone in the Baltic Sea. A second experiment investigated the metabolic acclimation of *G. vermiculophylla* to four temperatures, while comparing its physiological responses to those of two native species, *Fucus vesiculosus* and *Ulva lactuca*. This experiment showed that the optimum temperature for light-saturated photosynthesis increased for all three species as they became long-term acclimated to higher temperatures. Short-term incubation at high temperature (30°C) was suboptimal for all three algal species when grown at low temperatures (10–15°C) but the algae were unaffected when cultured at higher temperatures (20–25°C). Finally we evaluated the capacity of each of the three species for metabolic homeostasis and found that *F. vesiculosus* and *G. vermiculophylla* had an almost identical metabolic performance regardless of acclimation temperature, whereas net photosynthesis of *U. lactuca* increased significantly with growth temperature. Our results show that *G. vermiculophylla* shares traits with both the slow-growing, leathery *F. vesiculosus* (*K*-strategy) and the fast-growing, sheet-like *U. lactuca* (*r*-strategy), by combining relatively high growth rates with a robust metabolic response to changing temperatures. In conclusion, we suggest that having both *K* and *r* metabolic traits explains, in part, the invasion success of *G. vermiculophylla* in temperate estuaries.

Key words: alien species, Baltic Sea, *Fucus vesiculosus*, metabolism, non-indigenous species, temperature acclimation, *Ulva lactuca*

Introduction

Gracilaria vermiculophylla is a coarsely branched red algae that originates from the NE Pacific, but has been introduced to the E and W Atlantic and the W Pacific oceans, probably in association with oyster aquaculture (Saunders, 2009; Kim *et al.*, 2010). In the E Atlantic, *G. vermiculophylla* was first observed along the French coastline (Mollet *et al.*, 1998; Rueness, 2005). Following the first sighting in Europe, it has subsequently been found south of France, in Spain, Portugal, Italy and Morocco (Kim *et al.*, 2010; Sfriso *et al.*, 2010); and also northwards, in the North Sea and Wadden Sea, and penetrating into the Baltic Sea, where it is now present on the west coast of Sweden (Nyberg, 2006; Thomsen *et al.*, 2007; Nyberg *et al.*, 2009), in many of the Danish fjords in the Baltic area (Thomsen *et al.*, 2007, 2008), and in the bay of Kiel (Weinberger *et al.*, 2008).

The ecological impact of invasive species in marine systems is poorly understood (Ruiz *et al.*, 1999; Thomsen *et al.*, 2011) and it is possible the recent invasion of *G. vermiculophylla* into the Baltic Sea may have negative effects on the native flora and fauna. For example, *G. vermiculophylla* can smother sessile oyster communities (Thomsen & McGlathery, 2006) and have negative effects on seagrasses, particularly at high temperatures (Martinez-Luscher &

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Holmer, 2010; Höffle *et al.*, 2011). However, other studies suggest that the introduction of *G. vermiculophylla* into habitats with little substratum suitable for macroalgal attachment, may increase species diversity (Ruesink *et al.*, 2006; Nyberg *et al.*, 2009; Thomsen *et al.*, 2010; Thomsen, 2010).

To understand which environmental conditions control survival and growth, and thus the potential geographical distribution of *G. vermiculophylla*, studies have tested for the effects of stable (Rueness, 2005; Nejrup & Pedersen, 2012) and fluctuating (Nejrup & Pedersen, 2012) salinities, local grazing (Nejrup & Pedersen, 2010; Nejrup *et al.*, 2012) and nutrients (Tyler & McGlathery, 2006; Thomsen & McGlathery, 2007; Nejrup & Pedersen, 2010; Abreu *et al.*, 2011), and tolerances to sediment load and desiccation stress (Thomsen & McGlathery, 2007). However, it remains unclear why *G. vermiculophylla* has an advantage compared to native species of macroalgae in the shallow estuaries of Scandinavia. We hypothesized that the apparent success of *G. vermiculophylla* in shallow Scandinavian estuaries may, in part, be due to a high tolerance to extremes of, and changes in, light and temperature.

Growth of macroalgae is fundamentally regulated by temperature and light (Lobban & Harrison, 1994; Raikar *et al.*, 2001; Mendes *et al.*, 2012) and knowing a species' physiological response to these parameters is of vital importance to predict future distributions of non-indigenous macroalgae. Without quantitative data, predictive modelling is impossible (Kearney & Porter, 2009). Several studies have quantified the responses of *G. vermiculophylla* to manipulated levels of light and temperature in controlled laboratory experiments (Yokoya *et al.*, 1999; Raikar *et al.*, 2001; Rueness, 2005; Phooprong *et al.*, 2008; Abreu *et al.*, 2011; see also Table 5 for an overview); it should be noted that none of these studies developed predictive regression models for light and temperature responses. The algae used in these experiments were collected from several Japanese, one Portuguese and one French population of *G. vermiculophylla* and the results indicate that the species has a broad tolerance to many environmental variables. However, the presence of regional ecotypes within *Gracilaria* species (McLachlan & Bird, 1984) makes it less certain how Scandinavian populations of *G. vermiculophylla* will respond to these environmental variables. Growth in Scandinavian *G. vermiculophylla* populations has only been measured in field experiments. However, field experiments are characterized by uncontrolled co-varying factors such as day length, grazing rates and nutrient levels, making it difficult to identify temperature and light responses (Weinberger *et al.*, 2008; Nejrup & Pedersen, 2010). To understand the mechanisms that control growth of *G. vermiculophylla* in the Baltic region and to be able to generate quantitative and predictive performance models, controlled laboratory experiments that focus on the effects of light and temperature, and also potential interactive effects, are required. Using such data in, for example, spatial ecological modelling, may reveal important patterns relevant to the future distribution of *G. vermiculophylla* (Kearney & Porter, 2009).

Water temperature and light levels vary substantially from winter to summer in shallow Danish estuaries: they range from 0 to 27°C and from 0 to more than 600 μmol photons m^{-2} s^{-1} (Staehr & Borum, 2011). Tolerance of, and the ability to adapt to, these changing environmental conditions is likely to influence the ecological performance of *G. vermiculophylla* (and native co-existing algae) and may therefore also confer competitive advantages.

Algae can induce a range of biochemical adaptations to withstand changing light regimes at different water temperatures. At low temperatures, constraints on enzyme activity, membrane fluidity, and light and nutrient uptake reduce metabolic rates (Staehr & Wernberg, 2009) and growth (Lapointe *et al.*, 1984). As temperature increases, these constraints are gradually reduced, resulting in an increase in metabolic rates and nutrient demand, which also increases the likelihood of nutrient limitation. Beyond the optimum temperature, metabolic rates decline due to limitation in enzyme capacity (Atkin & Tjoelker, 2003) and there is reduced production of ATP due to constrained activity of Rubisco activase (Jensen, 2000). High temperatures eventually reduce metabolic performance through inactivation or denaturation of proteins. The ability to acclimate to changing temperature varies among species and plant types. For example, fast-growing microalgae can display significant capacity for physiological adjustment within a week (Staehr & Birkeland, 2006), compared to seasonal adjustments in slow-growing seagrasses (Staehr & Borum, 2011).

We conducted two experiments to test the temperature and light dependency of growth and metabolism in *G. vermiculophylla* over a wide range of temperature and light combinations. First we tested for interactive effects of light and temperature on somatic growth, using six light levels crossed with six temperatures. We hypothesized that minimum light requirements for growth are low for *G. vermiculophylla* but that these will increase at high temperatures to balance the enhanced respiratory costs. Second, we investigated in more detail the ability of *G. vermiculophylla* to acclimate its metabolic rates to increasing short-term incubation temperatures, and measured the associated changes in the tissue concentration of chlorophyll *a*, nitrogen and carbon. This experiment also included two native species with wide geographical ranges that overlap with that of *G. vermiculophylla* and that represent different form-functional groups (Littler & Littler, 1980) and growth strategies (Pedersen & Borum, 1996). These were the slow-growing, thick, leathery

alga *Fucus vesiculosus* and the fast-growing, sheet-forming alga *Ulva lactuca*, which represent archetypal *K* and *r* growth strategies, respectively. We hypothesized that the coarsely branched *G. vermiculophylla*, with its well-known broad environmental tolerances (e.g. Phooprong *et al.*, 2008), would have physiological traits in-between the typical *r* and *K* strategies. That is, *G. vermiculophylla* would have growth rates, and also an ability to adjust optimum temperatures and metabolic rates to different temperature regimes, that are higher than *F. vesiculosus* but lower than *U. lactuca.*

Materials and methods

Effect of light and temperature on growth – Experiment I

We first tested for interactive effects of water temperature and light on somatic growth of *Gracilaria vermiculophylla*. Algae were collected at Holckenhavn Fjord (Fynen, Denmark, 55°17.8′N, 10°46.2′E) in August 2007 (water temperature 16°C) and cultured for 6 weeks at 15°C under a 16 : 8 h light : dark cycle and *c.* 160 µmol photon m^{-2} s^{-1}. Algal thalli were exposed to six temperatures (5, 10, 15, 20, 25 or 30°C) fully crossed with six light levels (0, 9, 16, 34, 80 or 225 µmol photons m^{-2} s^{-1}). A total of 108 algal thalli (N = 3 for each light–temperature combination, each thallus having a blotted wet weight (WW) of 0.848 ± 0.308 g, mean ± sd) were incubated in separate 1.8-l clear plastic containers with ~ 1.7 l seawater. Light levels were controlled by placing different combinations of spectrally neutral black nylon-net over each 1.8-l container. The treatment receiving 0 µmol photons m^{-2} s^{-1} was wrapped in black plastic. The temperature was maintained using circulation pumps in one thermostat-controlled water bath per temperature treatment. Algal thalli were acclimated to each of the 36 combinations of temperatures and light for a minimum of 1 week. Water was diluted from natural seawater (30–35 psu, 1.3 mM total carbon at pH = 7.9) to 20 psu (frequently observed in Holckenhavn Fjord, from where the alga were collected, and a typical salinity for Danish waters in general) with tap water (2.5 mmol total carbon, pH = 8.3). The water was changed twice per week to maintain inorganic carbon levels. Five slow-release fertilizer pellets (PlataCore Depot 6M, Uranium Agrochem, Germany; total N = 14%, total P = 4%, mean weight = 0.14 ± 0.4 g) were added to each container to avoid nutrient limitation, and oxygen levels were maintained by air bubbling through air-stones. Blotted Wet Weight was determined two to three times per week during the 3-week experiment. Relative growth rate (µ) was calculated from:

$$\mu = \frac{\ln B_t - \ln B_0}{t} \qquad \text{Equation 1}$$

where B_0 and B_t are the initial and final Fresh Weight (FW) biomass between two successive measurements and t is the time that elapsed between these measurements (in days). µ has the unit day^{-1}.

Effect of temperature on cellular physiology – Experiment II

The effect of temperature acclimation on photosynthetic and respiratory performance was tested on *G. vermiculophylla, U. lactuca* and *F. vesiculosus. Ulva lactuca* and *F. vesiculosus* were collected at Nivå Harbour (Sealand, Denmark, 55°56.4′N, 12°31.6′E) and *G. vermiculophylla* at Holckenhavn Fjord during summer 2007. Individual algal thalli were incubated in 2.0-l glass containers with ~ 1.8 l seawater, placed in temperature-controlled water baths. For each species, four replicate experiments were conducted with an initial algal thallus biomass of 0.69 ± 0.20 g WW for *F. vesiculosus*, 0.99 ± 0.23 g WW for *G. vermiculophylla* and 3.18 ± 0.84 g WW for *U. lactuca* (in the form of 3-cm diameter circular disks). Before the experiment, all tissues were cleaned of epiphytes and then placed in a glass container receiving 150 µmol photons m^{-2} s^{-1} in a 16 : 8 light : dark cycle in 180 µm filtered seawater (20 psu) with a modified O_2 medium containing nitrogen, phosphorus and trace elements (Staehr & Birkeland, 2006). The water was changed every week to ensure nutrient-replete conditions.

The temperature was slowly changed (at a maximum of 5°C per day) from 14°C to a final experimental temperature of 10, 15, 20 or 25°C (N = 4). After reaching the final temperature (typically after 2 days), algae were long-term acclimated for 23 days at constant temperature. Wet weight was measured and two-thirds of the water exchanged twice per week. All other experimental parameters were maintained as described above during the 23-day period. Growth during this long-term acclimation was measured as in experiment I. Photosynthesis and respiration were measured during short-term incubations of each thallus at the end of the long-term experiment. Thallus segments (approximately 0.03 g dry weight) were cleaned of epiphytes prior to these short-term incubations in 25-ml glass bottles filled with GFC-filtered (1 µm) seawater. Dry weight was determined by drying thallus segments at 100°C to constant weight, which was typically 24 hours. Segments were small enough to ensure full light exposure and large enough to detect significant changes in dissolved oxygen. Bottles were placed on a rotating wheel and incubated in darkness and at saturating light (410 µmol photons m^{-2} s^{-1}). A rotating glass bean was added to each bottle to avoid diffusion limitation, and incubation duration was adjusted to keep changes in oxygen saturation below *c.* 50% within the incubation period. For each series of measurements, three control bottles without algae were incubated in darkness to account for background oxygen consumption. The control bottles were filled successively, so that one bottle was filled at the beginning, one bottle in the middle and one bottle at the end of the bottle-filling procedure for each series. Changes in oxygen concentrations were measured at 10, 20, 25 and 30°C (N = 4). Incubations ran for 45, 60, 90 and 90 min at 30, 25, 15 and 10°C, respectively, to ensure measurable changes in oxygen concentration. After incubation, bottles were kept in the dark at the experimental temperature to stop oxygen production while handling the other bottles. Oxygen concentrations were measured with a Clark-type oxygen microelectrode (OX500, Unisense; Aarhus, Denmark) and recorded using a picoammeter (PA2000, Unisense). In each temperature experiment, the electrode was calibrated to 0 and 100% of air saturation.

Total chlorophyll *a* was determined spectrophotometrically according to Jeffrey & Humphrey (1975). Organic carbon (C) and nitrogen (N) were measured using a Carlo–Erba EA–1108 CHN analyser (Carlo–Erba, Milano, Italy) according to the manufacturer's recommendations.

Curve fitting

Parameters describing the relationship between growth rate and light level in experiment I were obtained from a nonlinear regression fit of specific growth rate (μ) as a function of the photon flux density (E) according to a saturating exponential model (Webb *et al.*, 1974):

$$\mu = \mu_{max}(1 - exp(-\alpha E/\mu_{max})) + R \qquad \text{Equation 2}$$

where α is the initial slope of the curve (light-utilization parameter; d^{-1} mol^{-1} photons m^2), μ_{max} is the light-saturated growth rate (d^{-1}) and R is the growth rate in darkness. The light compensation level (E_C) was calculated as:

$$E_C = \mu_{max} \times log(1 + R/\mu_{max})/(-\alpha) \qquad \text{Equation 3}$$

The temperature responses of NP_{max} and $R_{thallus}$ (explained below) were fitted to the equations (equation 3) following Johnson *et al.* (1974), as modified by Staehr & Birkeland (2006). This formula assumes that a single temperature-sensitive enzyme, with active and inactive conformations, controls the fitted metabolic process (for its application to plant photosynthesis, see Santamaria & Van Vierssen, 1997):

$$Y = \mathrm{A}\frac{\mathrm{T}}{1+\mathrm{K}}\mathrm{e}^{-\mathrm{Ea}/(R_{gas}\cdot\mathrm{T})} \qquad \text{Equation 4}$$

where Y is NP_{max}, *or* $R_{thallus}$, A is a constant, E_a is the activation energy for the enzyme's conformational change (J mol^{-1}), R_{gas} is the gas constant (8.314 J mol^{-1} K^{-1}), T, the absolute temperature (in °K), and κ is the equilibrium constant of the reaction responsible for the enzyme's conformational change (mol). The latter depends on the absolute temperature according to equation (5):

$$k = \mathrm{e}^{-(\Delta H - \mathrm{T}\Delta S)/(R_{gas}\cdot\mathrm{T})} \qquad \text{Equation 5}$$

where ΔH and ΔS are the increase in enthalpy (J mol^{-1}) and entropy (J mol^{-1} K^{-1}), involved in the enzyme's conformational change. E_a/R_{gas} and $\Delta H/R_{gas}$ (both in °K) indicate the sensitivity of the rates to sub- and supraoptimal temperatures, respectively. This means that: (1) low E_a/R_{gas} values result in a sharp increase of rates with increasing temperatures, and (2) low $\Delta H/R_{gas}$ values result in a sharp drop in rates at temperatures above the optimum. The optimum temperature (T_{opt}) for NP_{max} and R_{dark} was determined by a Gaussian curve fit to each temperature–response curve. These temperature values were then used to calculate the corresponding maximum rates.

Statistical analysis

Two-way ANOVA was used to test for the effects of all experimental variables in experiment I and experiment II. In experiment I, we used a 2-way ANOVA to test for the effect of light and temperature on growth. In experiment II, we first used 2-way ANOVA to test for the effects of long-term temperature acclimation, between the three macroalgae species, on the response parameters of growth and the thallus concentration of chlorophyll *a*, carbon and nitrogen. Then we used 2-way ANOVA to test for interactive effects of long-term acclimation temperature and short-term incubation temperature on net photosynthesis and dark respiration. These analyses were carried out separately for all three species. Tukey's test was used to compare pairs of mean when ANOVA identified significant differences. A Kolmogorov–Smirnoff goodness-of-fit test was used to test for normality and Levene's test was used to test for homogeneity of variances. Data were ln-transformed when necessary to obtain normally distributed data and equal variances. All statistical analyses were carried out using SYSTAT (Systat Software, Chicago, IL, USA).

Results

Effect of light and temperature on growth – Experiment I

The growth rate of *G. vermiculophylla* increased significantly with light at all temperatures, but there was a strong interaction in which the light dependency of growth differed significantly between experimental temperatures (Fig. 1, Table 1). Growth rates showed a saturating relationship to light for all experimental temperatures (Fig. 1A, B, all P values <0.01). Growth increased significantly with temperature and light, and the highest growth rate (0.045 ± 0.001 day^{-1}, mean ± SD) was observed for the combination of 20°C and 225 µmol photons day^{-1} s^{-1}. In the dark treatments there was no net growth or appreciable loss, demonstrating that *G. vermiculophylla* can maintain almost constant biomass, even at high temperatures, after 3 weeks of darkness. In the 5°C treatment, *G. vermiculophylla* had a low overall growth rate regardless of the light level (Fig. 1A), which was significantly lower than at all other temperatures (Tukey test: P <0.000). The relationship between the maximum specific growth rate and temperature was bell-shaped (Fig. 2A), with a fitted optimum of 21°C. A similar unimodal pattern was found for light utilization efficiency (α; Fig. 2B), although this peaked slightly higher, at 23°C (~0.3 d^{-1} mol^{-1} photons m^{-2}). However, the pattern for the light compensation levels (E_C; Fig. 2C) was different; E_C was low from 5 to 20°C (<1 µmol photons m^{-2} s^{-1}) but significantly higher at 25°C (4 µmol photons m^{-2} s^{-1}) and 30°C (7 µmol photons m^{-2} s^{-1}). Thus, the five-fold increase in E_C in the 30°C treatment, compared with the 0 to 20°C treatments, indicated that the increase in respiration exceeds the increase in photosynthesis with increasing temperature.

Effect of temperature on growth and cellular physiology – Experiment II

All alga species showed a unimodal dependency of light-saturated growth on increasing temperature, with

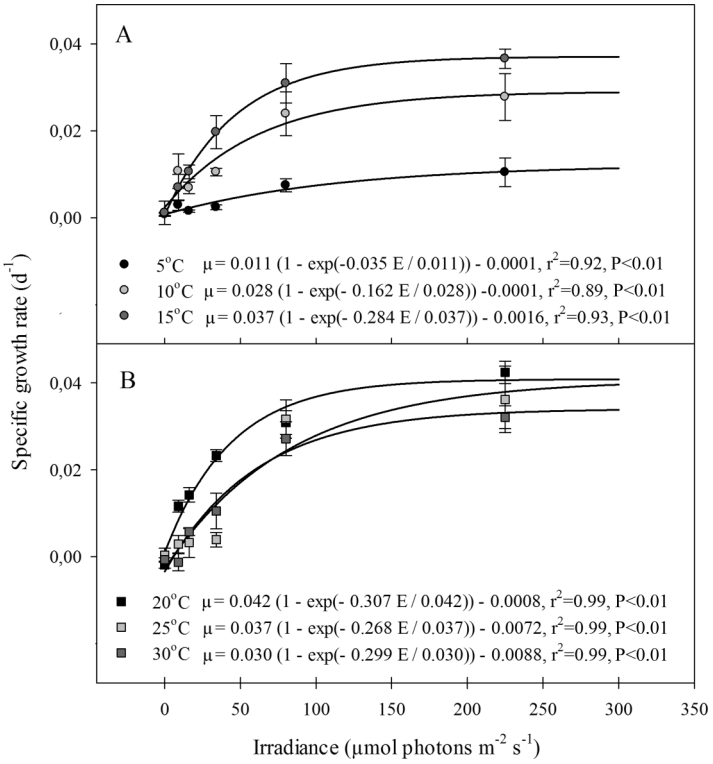

Fig. 1. Experiment I: Specific growth rates as a function of irradiance of *G. vermiculophylla* at (A) 5, 10 and 15ºC and (B) 20, 25 and 30ºC. Equations for each curve are shown. See text for explanation of equation 1. Errors are SE, $N = 3$.

Table 1. *Gracilaria vermiculophylla*: ANOVA for the effects of temperature and light and interactions between the two factors for experiment I and experiment II. C = organic carbon; N = Nitrogen; C : N = weight ratio of C and N; and Chl *a* = Chlorophyll *a*. Bold indicates significant result.

Experiment	Response	Factor	df	MS	*F*-Ratio	*P*
I	growth	temperature	5	0.001	20.227	**<0.001**
		light	5	0.003	108.899	**<0.001**
		temp. × light	25	0.000	4.096	**<0.001**
		error	72	0.000		
II	growth	temperature	3	0.000	6.260	**0.002**
		species	2	0.002	33.104	**<0.001**
		temp. × spec.	6	0.000	1.294	0.285
		error	36	0.000		
II	C	temperature	3	0.016	10.181	**<0.001**
		species	2	0.004	2.813	0.073
		temp. × spec.	6	0.004	2.521	**0.039**
		error	36	0.002		
II	N	temperature	3	44.150	27.592	**<0.001**
		species	2	2.911	1.820	0.177
		temp. × spec.	6	16.147	10.091	**<0.001**
		error	36	1.600		
II	C : N	temperature	3	367.020	15.394	**<0.001**
		species	2	5.437	0.228	0.797
		temp. × spec.	6	166.975	7.004	**<0.001**
		error	36	23.841		
II	Chl *a*	temperature	3	0.028	0.820	0.491
		species	2	17.130	493.726	**<0.001**
		temp. × spec.	6	0.224	6.443	**<0.001**
		error	36	0.035		

optimum temperatures at around 20°C (Fig. 3). Growth increased significantly with temperature from 10°C to 20°C (2-way ANOVA: $F = 6.30$, $P = 0.002$) with species having significantly different growth rates (2-way ANOVA: $F = 33.10$, $P = 0.0001$) but no interaction effect (2-way ANOVA: $F = 1.294$, $P = 0.285$). While *G. vermiculophylla* and *U. lactuca* had almost identical growth rates,

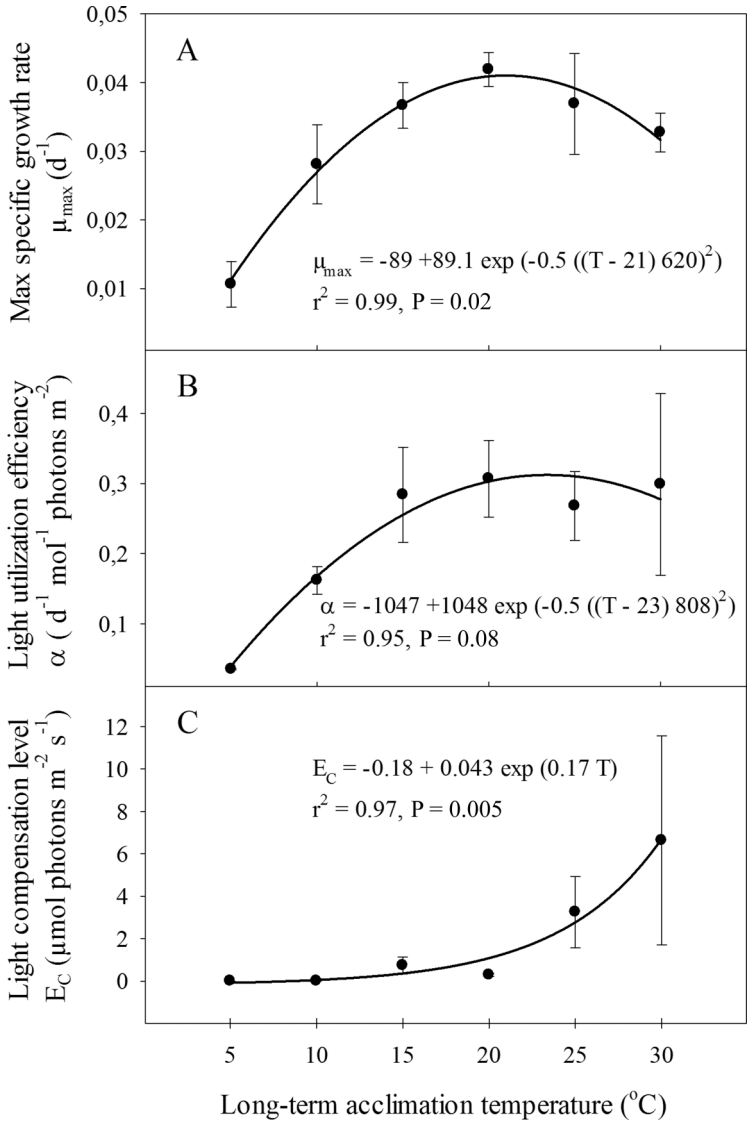

Fig. 2. Experiment I: Parameters derived from models of growth rate vs light curves. (A) Light-saturated growth rate (μ_{max}); (B) initial slope of the growth vs. light curve (α) and (C) minimum light demand (E_C) to maintain a net positive growth rate. Errors are SE, $N = 3$. Curves in A and B are fitted using a Gaussian model and C with an exponential growth model. According to the Gaussian curve-fits, optimum temperatures for μ_{max} and α were 21ºC and 23ºC respectively.

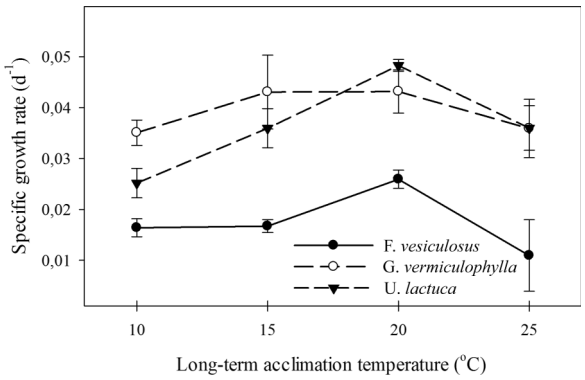

Fig. 3. Experiment II: Specific growth rates for three macroalgal species in response to four growth temperatures. Errors are SE, $N = 4$.

F. vesiculosus had growth rates half that of the other two species, with the lowest rate found at 25°C (~ 0.01 day^{-1}).

Significant interactions were observed for all cell physiology parameters (C, N, C : N and chlorophyll *a*: Table 1), indicating that temperature responses varied between the three species. Cellular chlorophyll *a* was generally highest for *F. vesiculosus*, lowest for *U. lactuca* and intermediate for *G. vermiculophylla* when comparing all temperatures (Table 2). The cellular content of nitrogen decreased with increasing temperature for *G. vermiculophylla* and *U. lactuca* but remained relatively constant for *F. vesiculosus* across temperatures. Cellular carbon content decreased with increasing temperature when comparing all species (Tables 1 and 2). However, we found no significant effect of temperature for the individual species (1-way ANOVA, $P > 0.05$). Finally, we found a significant interaction between temperature and species ($F = 7.004$, $P < 0.001$) for the weight ratio of carbon to nitrogen (C : N), which increased with increasing temperature for *G. vermiculophylla*

Table 2. Experiment II: cellular characteristics of *Fucus vesiculosus, Gracilaria vermiculophylla* and *Ulva lactuca* long-term acclimated to four different growth temperatures. Measurements (mean ± SD, $N = 4$) were made of chlorophyll *a* (Chl *a*; µg g^{-1} DW), organic carbon (C; mg g^{-1} DW) and nitrogen (N; mg g^{-1} DW), and the weight ratio of C and N.

	Acclimation temperature (°C)	Chl *a*	C	N	C : N
Fucus vesiculosus	10	2.94 ± 0.87	321.90 ± 4.74	9.15 ± 1.13	35.70 ± 4.22
	15	3.30 ± 0.23	322.93 ± 1.95	8.93 ± 1.16	36.80 ± 4.95
	20	3.35 ± 0.30	323.23 ± 4.07	9.72 ± 1.03	33.64 ± 3.75
	25	2.4 ± 0.31	312.23 ± 8.09	9.67 ± 0.59	32.39 ± 1.57
Gracilaria vermiculophylla	10	1.04 ± 0.15	334.63 ± 1.13	14.00 ± 0.76	23.98 ± 1.23
	15	0.97 ± 0.12	323.75 ± 7.18	12.16 ± 0.28	26.65 ± 0.95
	20	1.12 ± 0.09	294.20 ± 27.31	7.65 ± 0.98	38.69 ± 1.66
	25	0.78 ± 0.08	299.18 ± 12.85	7.01 ± 1.62	44.86 ± 9.48
Ulva lactuca	10	0.44 ± 0.07	319.73 ± 4.27	12.43 ± 1.56	26.14 ± 3.23
	15	0.37 ± 0.08	320.10 ± 5.97	11.34 ± 1.74	28.87 ± 4.14
	20	0.26 ± 0.03	308.13 ± 5.28	8.11 ± 0.16	38.00 ± 0.45
	25	0.48 ± 0.04	293.28 ± 4.85	6.65 ± 0.79	44.81 ± 5.74

and *U. lactuca* (almost identical) but decreased slightly for *F. vesiculosus*.

Temperature dependency and sensitivity of metabolic rates – Experiment II

We found a general tendency for light-saturated photosynthesis (NP_{max}) and dark respiration (R_{dark}) to increase for all algae species as a function of short-term incubation temperature (Figs 4 and 5). The 'cold' (i.e. 10 and 15°C) and 'warm' (i.e. 20 and 25°C) long-term acclimated algae tended to respond differently to short-term incubations at increasing temperatures (Fig. 4). The effect on NP_{max} and R_{dark} of long-term acclimation temperature and short-term incubation temperature (see Table 3) was significant for all species ($P < 0.001$, Table 4). Furthermore, a significant short-term × long-term temperature interaction effect was observed for all species except with respect to the respiratory response of *U. lactuca* ($F = 0.675$, $P = 0.727$). This interaction effect indicated, with the exception of *U. lactuca,* that the response of the algae to increasing short-term incubation temperature was significantly affected by the temperature to which the algae had been long-term acclimated. At low short-term incubation temperature (10°C), the photosynthetic rates of species that were long-term acclimated to cold temperatures were generally higher than those that were long-term acclimated to warm temperature. In comparison, at high incubation temperature (30°C) these cold-acclimated algae generally had lower photosynthetic responses than the warm-acclimated algae. Exceptions to this pattern were the unexpectedly high photosynthetic rates of *U. lactuca* and *F. vesiculosus* that had been long-term acclimated to 25°C (Fig. 4A, C). The suboptimal conditions for cold-acclimated algae at high temperatures were also seen in elevated respiration rates, except in *F. vesiculosus* (Fig. 5A). Warm-acclimated algae generally displayed lower respiration rates than the cold-acclimated (Fig. 5).

The optimum temperature for light-saturated net photosynthesis (T_{opt} of NP_{max}) varied significantly between species and between long-term acclimation temperatures ($F = 5.620$, $P < 0.001$); this interaction occurred because T_{opt} increased for *G.*

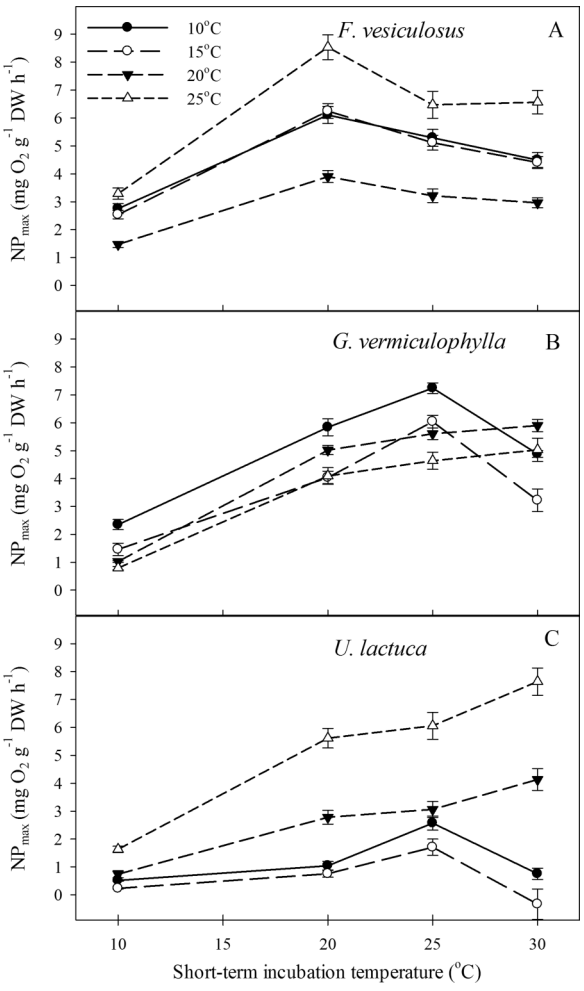

Fig. 4. Experiment II: Light-saturated net photosynthesis of short-term incubations to different long-term acclimation temperatures of (A) *F. vesiculosus*; (B) *G. vermiculophylla* and (C) *U. lactuca*. Errors are SE, $N = 4$.

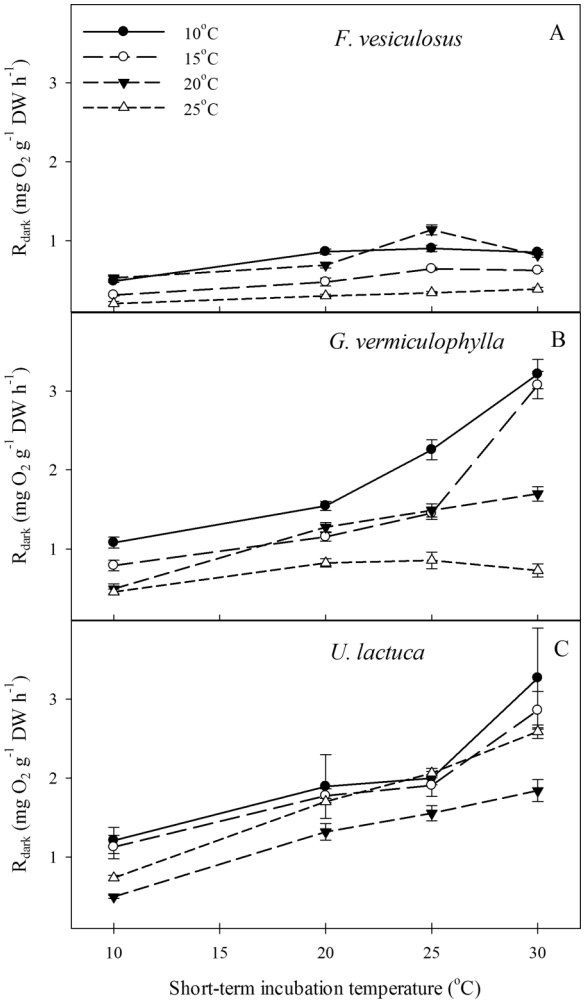

Fig. 5. Experiment II: Dark respiration rates of short-term incubations to different long-term acclimation temperatures of (A) *F. vesiculosus*; (B) *G. vermiculophylla* and (C) *U. lactuca*. Errors are SE, $N = 4$.

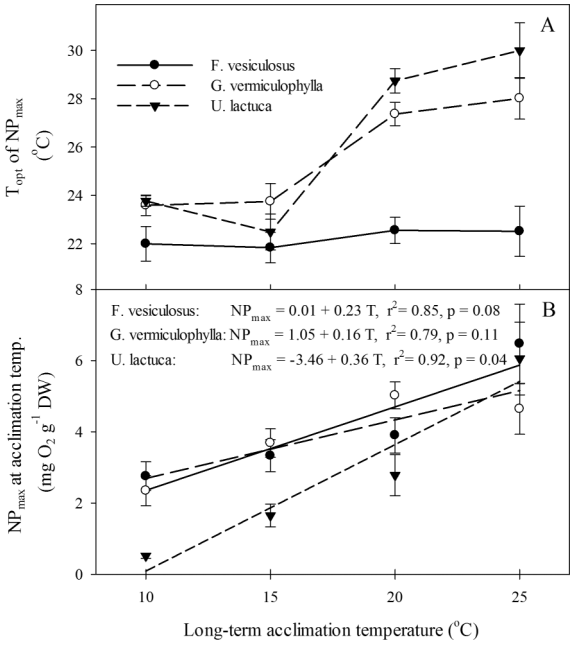

Fig. 6. Experiment II: Metabolic adjustments for three macroalgae species acclimated to four growth temperatures. (A) Optimum temperature of light-saturated net photosynthesis (NP_{max}), and (B) NP_{max} values at short-term incubation temperatures corresponding to the same long-term acclimation temperatures. Errors are SD, $N = 4$.

vermiculophylla and *U. lactuca*, but not for *F. vesiculosus* (Fig. 6A). At the highest acclimation temperature (25°C), *G. vermiculophylla* and *U. lactuca* had optimum temperatures of 25.7 ± 2.2°C (mean ± SD) to 26.3 ± 3.9°C, respectively, which were not significantly different from each other (Tukey's test, $P = 0.502$). In comparison, *F. vesiculosus* had a relatively constant T_{opt} of *c*. 22°C (Fig. 6A), which was significantly lower than for the two other species (Tukey's test, $P < 0.0001$). Thus, while *G. vermiculophylla* and *U. lactuca* displayed a strong ability to adjust their optimum temperature for photosynthesis towards the long-term acclimation temperature, metabolic changes for *F. vesiculosus* were less pronounced.

To further evaluate the ability to adjust metabolism to prevailing growth temperatures, we compared the rates of light-saturated net photosynthesis between algae that had been long-time acclimated and short-term incubated at the same temperature (i.e. an algae long-term acclimated at 10°C was short-term incubated at 10°C; Fig. 6B). Algae acclimated to a given growth temperature are expected to optimize their cellular physiology (pigmentation, carbon hydrates, enzyme concentration) to the prevailing growth conditions. As a result, full acclimation (metabolic homeostasis) should result in similar net photosynthetic rates across a range of acclimation temperatures (Davison *et al.*, 1991). In accordance with this, we would expect the slope of the regression line describing net photosynthetic rates at corresponding long-term acclimation and short-term incubation temperatures to be zero for a species with full ability to acclimate. NP_{max} increased with increasing temperature for all three species (Fig. 6B) but the slope was significantly different from zero only in *U. lactuca*; though insignificant, the trend was similar in *G. vermiculophylla* ($P = 0.11$) and *F. vesiculosus* ($P = 0.08$). Consequently, *U. lactuca* had much lower photosynthetic rates at lower temperatures than *G. vermiculophylla* and *F. vesiculosus*, suggesting a higher degree of metabolic homeostasis in the two latter species.

The sensitivity of metabolism to suboptimal temperatures can be evaluated using the E_a/R_{gas} expression, which describes the initial slope of the fitted curve of temperature-dependent photosynthesis. For *G. vermiculophylla* and *F. vesiculosus*, E_a/R_{gas} ratios for NP_{max} tended to increase with increasing temperature (Table 3) indicating that the warm-acclimated algae had a weaker photosynthetic response to increasing temperature than cold-acclimated algae. In contrast, *U. lactuca* showed an opposite change in the E_a/R_{gas} ratio, indicating an even stronger response to short-term

Table 3. Experiment II: parameter estimates from temperature response curves of light-saturated net photosynthesis (NP_{max}) and dark respiration (R_{dark}) of *Fucus vesiculosus, Gracilaria vermiculophylla* and *Ulva lactuca* long-term acclimated to different growth temperatures. E_a/R_{gas} is the activation energy/gas constant and $\Delta H/R_{gas}$ the increase in entropy/gas constant, which are coefficients in the Johnson *et al.* (1974) temperature response model. Values are mean ± SD ($N = 4$).

Species	Acclimation temperature (°C)	$E_a/R\ NP_{max}$	$E_a/R\ R_{dark}$	$\Delta H/R\ NP_{max}$	$\Delta H/R\ R_{dark}$
Fucus vesiculosus	10	11.30 ± 0.05	11.52 ± 0.10	18.87 ± 0.66	14.47 ± 1.77
	15	11.37 ± 0.05	11.50 ± 0.16	19.82 ± 0.41	11.21 ± 2.41
	20	11.46 ± 0.09	11.46 ± 0.08	19.17 ± 1.35	13.52 ± 1.52
	25	11.25 ± 0.07	7.43 ± 4.64	18.30 ± 1.01	6.58 ± 6.40
Gracilaria vermiculophylla	10	11.20 ± 0.05	6.91 ± 2.55	26.86 ± 3.05	2.14 ± 3.84
	15	11.36 ± 0.06	8.62 ± 0.70	35.52 ± 0.86	0.60 ± 0.64
	20	11.57 ± 0.04	11.63 ± 0.15	18.84 ± 1.25	13.49 ± 2.39
	25	11.63 ± 0.08	12.00 ± 0.65	18.74 ± 1.84	13.14 ± 7.55
Ulva lactuca	10	11.85 ± 0.28	8.95 ± 3.30	36.01 ± 0.14	5.52 ± 5.47
	15	11.75 ± 0.13	6.49 ± 2.94	35.90 ± 0.34	3.02 ± 4.03
	20	11.43 ± 0.12	11.57 ± 0.10	12.67 ± 7.36	12.37 ± 2.20
	25	11.32 ± 0.03	11.38 ± 0.06	12.97 ± 1.25	10.02 ± 1.47

Table 4. Experiment II: 2-way ANOVA for the interaction effects of acclimation temperature and incubation temperature of light-saturated photosynthesis (NP_{max}), dark respiration (R_{dark}) and the metabolic adjustments (Topt *NP*max) for three macroalgal species.

Response	Species	Factor	df	MS	*F*-Ratio	*P*
NP_{max}	*Gracilaria vermiculophylla*	acclimation temperature	3	7.357	22.605	<0.001
		incubation temperature	3	60.043	184.492	<0.001
		acclimation temperature × incubation temperature	9	2.369	7.280	<0.001
		error	48	0.325		
	Fucus vesiculosus	acclimation temperature	3	29.593	68.708	<0.001
		incubation temperature	3	37.812	87.791	<0.001
		acclimation temperature × incubation temperature	9	1.046	2.428	0.023
		error	48	0.431		
	Ulva lactuca	acclimation temperature	3	67.524	141.628	<0.001
		incubation temperature	3	20.987	44.018	<0.001
		acclimation temperature × incubation temperature	9	6.334	13.286	<0.001
		error	48	0.477		
R_{dark}	*Gracilaria vermiculophylla*	acclimation temperature	3	4.961	101.479	<0.001
		incubation temperature	3	6.084	124.444	<0.001
		acclimation temperature × incubation temperature	9	0.889	18.173	<0.001
		error	48	0.049		
	Fucus vesiculosus	acclimation temperature	3	0.409	81.015	<0.001
		incubation temperature	3	0.409	81.015	<0.001
		acclimation temperature × incubation temperature	9	0.042	8.317	<0.001
		error	48	0.005		
	Ulva lactuca	acclimation temperature	3	1.831	7.274	<0.001
		incubation temperature	3	8.235	32.723	<0.001
		acclimation temperature × incubation temperature	9	0.170	0.675	0.727
		error	48	0.252		
TopT *NP*max	All	acclimation temperature	3	53.824	26.604	<0.001
		incubation temperature	2	75.916	37.523	<0.001
		acclimation temperature × incubation temperature	6	11.371	5.620	<0.001
		error	36	2.023		

temperature increases in warm- compared to cold-acclimated algae. To evaluate temperature responses of photosynthesis at supraoptimal temperatures (above T_{opt}) we calculated the $\Delta H/R_{gas}$ (enthalpy/gas constant) ratio. This showed a general decrease for all three species with increasing growth temperature, indicating a general rise in NP_{max} rates at supraoptimal temperatures (Table 2). In other words, algae acclimated to high temperatures could better tolerate supraoptimal temperatures than cold-acclimated algae. No clear trends in the temperature sensitivity of respiratory metabolism were found for any of the species.

Discussion

The potential success of a non-indigenous species may be predicted by the extent of the species' native geographical range (Forcella & Wood, 1984; Goodwin *et al.*, 1998): the larger the native range of the species, the more likely the species is to be able to

Table 5. Laboratory studies of temperature dependence of somatic growth, light-saturated photosynthesis (NP_{max}) and respiration rates. If the authors worked with both gametophyte and tetrasporophytes, we show data only for tetrasporophytes (Yokoya *et al.*, 1999; Abreu *et al.*, 2011). E_c = the minimum light requirement (µmol photons m^{-2} s^{-1}). NA = data not available. Studies marked with an asterisk include factorial-designed experiments to test for interaction of temperature with light and/or salinity. Studies marked with # indicate where temperature dependencies were parameterized.

Study	Experimental conditions	Region	Growth rate (day^{-1})	NP_{max} (mg O_2 g^{-1} DW h^{-1})	Respiration (mg O_2 g^{-1} DW h^{-1})
Present (Experiment I)*#	Temperature: 6 levels, 5–30°C Light: 6 levels; 0–225 µmol photons m^{-2} s^{-1} Salinity 20 psu Photoperiod: 16 : 8	Scandinavia	0.001–0.045 T_{opt}: 21°C E_c: 1–7	NA	NA
Present (Experiment II)*#	Temperatures: four long-term acclimation Light: 0 and 410 µmol photons m^{-2} s^{-1} temperatures, 10–25°C; four short-term incubation temperatures, 10–30°C Salinity 20 psu Photoperiod: 16 : 8	Scandinavia	0.035–0.043 T_{opt}: 15–20°C	0.8–7.2 T_{opt}: 23.6–28.0°C	0.4–3.2 T_{opt}: 25–30°C
Abreu *et al.* (2011)*	Temperature: four levels, 10–25°C Light: 40 or 100 µmol photons m^{-2} s^{-1} Salinity 33 psu Photoperiods: 8 : 16, 12 : 12, 16 : 8	Portugal	0.025–0.064 T_{opt}: 20°C	NA	NA
Phooprong *et al.* (2008)*	Temperature: 7 levels, 5–35°C Light: 0–710 µmol photons m^{-2} s^{-1} Salinity 10, 20, 33 psu Photoperiod: 16 : 8	Japan	NA	0.8–15.1 T_{opt}: 25–30°C E_c: 1–59	0.005–1.0 T_{opt}: 35°C
Rueness (2005)*	Temperature: 15.5, 19.5, 25°C Light: 40 or 100 µmol photons m^{-2} s^{-1} Salinity 10, 20, 30 psu Photoperiod: 16 : 8	Sweden, France	0.03–0.075 T_{opt}: 20	NA	NA
Raikar *et al.* (2001)	Temperature: 4 levels, 10, 20, 25, 30°C Light: 10, 40, 80 µmol photons m^{-2} s^{-1} Salinity 30 psu Photoperiod: 12 : 12	Japan, India, Malaysia	0.03–0.12 T_{opt}: 20–25°C	NA	NA
Yokoya *et al.* (1999)	Temperature: 6 levels, 5–30°C Light: 5 levels, 20–100 µmol photons m^{-2} s^{-1} Salinity 30–32 psu Photoperiod: 14 : 10	Japan	0.015–0.10 T_{opt}: 20°C	NA	NA

survive along environmental stress gradients, e.g. along salinity, temperature and light gradients. The native range of *Gracilaria vermiculophylla* is the SW Pacific Ocean, where physical parameters vary greatly on regional and seasonal scales (Phooprong *et al.*, 2008), and we therefore expected it to exhibit high tolerance to environmental stress. Results from our experiments support this idea and indicate a plastic response to a wide range of combinations of light and temperature conditions. Our findings thereby support other physiological studies that have tested how *G. vermiculophylla* respond to temperature and light stress (Table 5).

Importantly, we found many significant interaction effects of temperature and light on growth, metabolism and chlorophyll *a* content (Table 1), implying that the physiological responses of *G. vermiculophylla* should always be interpreted in the context of explicitly listed temperature and light conditions. A combination of low light and high temperature can limit the ecological performance of temperate macroalgae during summer months, particular at night, in turbid water, and at depth or below algal mats or seagrass canopies (Peckol & Rivers, 1996). However, *G. vermiculophylla* is able to grow in very low light conditions (<1 μmol photons m^{-2} s^{-1}) and at high temperature (up to at least 25°C; at 30°C, high respiration overtook photosynthesis). The low light levels required to maintain positive biomass production partially explain the high standing biomass in shallow estuaries; here *G. vermiculophylla* is often found in thick mats (Thomsen *et al.*, 2006; Nyberg *et al.*, 2009; Nejrup & Pedersen, 2010) in which significant self-shading occurs (Krause-Jensen *et al.*, 1996; Peckol & Rivers, 1996). Our findings suggest that *G. vermiculophylla* should generally be able to sustain positive growth in shallow Scandinavian estuaries during all seasons (except for the low saline parts (<5 PSU) of the Baltic Sea). This is in contrast to the findings of Weinberger *et al.* (2008) who performed *in situ* experiments and found positive growth rates in *G. vermiculophylla* during the summer (high temperature and high light) but not during spring and autumn, when there was reduced light and temperature. However, biomass loss caused by mesoherbivores and fragmentation (potentially induced by the grazers) in the field experiment may explain this discrepancy. Indeed, Weinberger *et al.* (2008) recorded higher grazing rates than were reported by Nejrup & Pedersen (2010). Low temperatures and ice cover (i.e. low light) have been proposed to limit the distribution of *G. vermiculophylla* in the Baltic Sea (Nyberg *et al.*, 2009) but in the present experiment, we found positive growth rates at conditions that are relatively similar to those found under ice (1 μmol photons m^{-2} s^{-1} and 5°C: Leu *et al.*, 2010; Smethie *et al.*, 2011). Our results are consistent with the findings of Yokoya *et al.* (1999, see Table 5), who also found positive growth rates at a temperature of 5°C in culture experiments. This indicates that *G. vermiculophylla* can survive and even grow a little in cold periods (down to 5°C) if not affected by other stressors. Even though positive growth rates were observed at 5°C, germination is greatly impaired at this temperature (Abreu *et al.*, 2011), which could reduce recruitment success and potentially the spread of the species to regions with low summer temperatures. However, *G. vermiculophylla* is not necessarily dependent on sexual reproduction because, like other *Gracilaria* species, it can reproduce asexually through fragmentation (e.g. Rueness, 2005).

Both *G. vermiculophylla* and the two native species increased their long-term acclimated photosynthetic rates when exposed to similar short-term incubation temperatures, but with a stronger increase for *U. lactuca* (Fig. 4), which suggests a less pronounced ability to acclimate net photosynthesis for this species compared with the two slower-growing perennial algae. Similar results for the large brown alga *Laminaria saccharina* were shown by Davison *et al.* (1991) and explained by acclimation of Calvin cycle enzyme activities, which enables algae grown at different temperatures to achieve similar rates of light-saturated photosynthesis (Davison & Davison, 1987).

The optimum growth temperature of *G. vermiculophylla* was found to be *c*. 20°C (experiment I) supporting several other studies (Yokoya *et al.*, 1999; Rueness, 2005; Abreu *et al.*, 2011; Table 5), even though differences in experimental setup can make direct comparisons difficult. However, Yokoya *et al.* (1999) found somewhat higher growth rates of *G. vermiculophylla* than those in this study, probably as a result of differences in experimental design (e.g. Yokoya measured growth in small apical fragments that did not self-shade and contained a high biomass proportion of actively growing tissue; such material typically shows high growth rates). Interestingly, Phooprong *et al.* (2008) found that the optimum temperature of different native Japanese populations of *G. vermiculophylla* varied with local temperature regimes, suggesting that different ecotypes have different temperature tolerances. The notion of different ecotypes has also been suggested for other *Gracilaria* species (McLachlan & Bird, 1984) and for different invasive *G. vermiculophylla* strains in Europe (Rueness, 2005), and may explain the relatively low growth rates observed here.

Prior to the experiment, we anticipated that growth and photosynthetic rates of the three species in experiment II would be related to their form-functional grouping, i.e. *U. lactuca* >> *G.*

vermiculophylla > *F. vesiculosus* (Littler & Littler, 1980). Our results partly supported this hypothesis for growth rates, but net photosynthesis was only marginally different between *U. lactuca* and *G. vermiculophylla*. This could indicate that *U. lactuca*, despite frequent water exchanges, experienced nutrient limitation at high temperatures, a notion supported by the tissue nitrogen data which showed decreasing concentration of N with increasing acclimation temperature. Pedersen & Borum (1996) suggested that N-limitation for *U. lactuca* occurs when cellular concentrations decrease below 2.17% of DW with a subsistence quota of 0.71%, below which growth ceases. Similar levels for *F. vesiculosus* provide N-limited growth below 1.71% of DW with a subsistence quota of 0.55%. In this study, the content of N varied from 0.66 to 1.24% (at 25 and 10°C respectively) for *U. lactuca* and 0.89 to 0.99% for *F. vesiculosus* (no temperature dependency). While we do not have comparative data for *G. vermiculophylla*, the measured N-content (0.70 to 1.40%, at 25 and 10°C respectively) was lower than the average seasonal values that have been reported, of approximately 2% (Nejrup & Pedersen, 2010). These results indicate that despite our efforts to maintain N-replete conditions, all three species could have been N-limited; although this is probably most pronounced for *U. lactuca* at high temperatures (as this species has by far the highest maximum growth rate and nitrogen demand: Pedersen & Borum, 1996). However, reduced tissue nitrogen content amongst algae acclimated to warm conditions does not automatically imply nutrient-limited growth. For example, cold-acclimated plants can have a high N content as a result of high tissue concentrations of enzymes (Raven & Geider, 1988), lipid content (Raison *et al.*, 1980), and pigment concentration (Falkowski & Laroche, 1991) to reduce low temperature stress. Nevertheless, as all three species were grown under identical conditions, direct comparisons of temperature-acclimated metabolism between the species remain possible. Future investigations of temperature acclimatization in respiration and photosynthesis of macroalgae, however, should also evaluate the potential impact of nitrogen limitation, which previously has been shown to influence metabolic performance (Raven & Geider, 1988).

Long-term acclimation to 25°C reduced the growth rate of all algal species when compared with rates at 20°C, although this decrease was most pronounced for *F. vesiculosus* (Fig. 3). In addition, all three species showed optimum growth at 20°C, somewhat higher than reported by Fortes & Lüning (1980) for *U. lactuca* (10–15°C) and *F. vesiculosus* (15°C). We discovered that light and temperature have interactive effects on growth (and therefore should be quantified and reported), but Fortes & Lüning did not report light levels for their experiment; a direct comparison is therefore difficult and the reported differences could thus be due to different culture conditions. Furthermore, Nygaard & Dring (2008) reported optimum growth for *F. vesiculosus* comparable to this study at long-term temperatures down to 15–20°C. Overall these differences in optimum temperatures suggest the presence of regional ecotypes that are adapted to prevailing environmental conditions.

Our laboratory experiments suggest that *G. vermiculophylla* shares common traits with both the slow-growing, leathery *F. vesiculosus*, which can be classified as a *K*-strategist, and the fast-growing, sheet-forming *U. lactuca*, which can be regarded as an *r*-strategist (Littler & Littler, 1980; Thomsen & McGlathery, 2007). When comparing temperature dependencies, *G. vermiculophylla* had an almost identical, but marginally weaker response to higher temperatures than *U. lactuca*; this was observed for somatic growth, photosynthesis and respiration. Furthermore, the calculated optimum temperature for net photosynthesis at the temperature at which the algae had long-term acclimated, was more or less the same for the two species. In contrast, the biochemical analysis of thallus content grouped *G. vermiculophylla* together with *F. vesiculosus*, being significantly different from that of *U. lactuca*; this difference was also seen when comparing the species' efficiency in acclimating to changing temperatures. In conclusion, we suggest that one plausible explanation for the success of *G. vermiculophylla* in Scandinavian estuaries is, at least in part, the combination of relatively high growth rates with a robust metabolic response to both low and high light levels, and to varying temperatures.

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