

Tolerances to hypo-osmotic and temperature stresses in native and invasive species of *Gracilaria* (Rhodophyta)

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ABSTRACT: Physiological responses to the hypo-osmotic and temperature stresses of an invasive species, *Gracilaria vermiculophylla*, were compared to those of the native Atlantic species, *G. tikvahiae* and *G. cervicornis*. For the hypo-osmotic and lower lethal temperature experiment, a Connecticut (CT) strain of the invasive *G. vermiculophylla* and a Rhode Island (RI) strain of the native *G. tikvahiae* were cultivated at combinations of five different salinities (S = 5, 15, 20, 25 and 30) and at five different temperatures (5°C, 10°C, 15°C, 20°C and 25°C) for 3 weeks. For the upper lethal temperature experiment, two strains of *G. vermiculophylla* from CT and Portugal, two strains of *G. tikvahiae* from CT and RI and one Florida strain of *G. cervicornis* were cultivated at temperatures ranged from 22°C to 39°C in 2°C or 3°C increments for 14 days. *Gracilaria vermiculophylla* showed a wide range of temperature (5°C–34°C) and salinity (5–30 S) tolerance; whereas, *G. tikvahiae* cannot withstand harsher environmental stresses, such as prolonged exposures to salinities of ≤ 20 S and temperatures of $\leq 10^\circ\text{C}$ or $\geq 34^\circ\text{C}$. *Gracilaria vermiculophylla* also grew faster and had higher survival rates than *G. tikvahiae* or *G. cervicornis*. These results suggest that the high tolerance and growth capacity of *G. vermiculophylla* may be responsible for the successful invasion of this alga into Long Island Sound and elsewhere along the east coast of North America.

KEY WORDS: *Gracilaria*, Hypo-osmotic stress, Invasive species, Climate change, Temperature

INTRODUCTION

Seaweeds have invaded many coastal ecosystems around the world. These invasive species have displaced native species and have transformed the diversity and functioning of local marine ecosystems. As with many successful marine invaders, *Gracilaria vermiculophylla* (Ohmi) Papenfuss has broad tolerances to temperatures, salinities, nutrients, sediment burial and grazing (Thomsen & McGlathery 2007; Nyberg *et al.* 2009; Abreu *et al.* 2011; Nettleton *et al.* 2013; Hu & Juan 2014; Hammann *et al.* 2016). This species also grows readily from fragments (Nyberg & Wallentinus 2009; Abreu *et al.* 2011) and perennating discs, and is capable of successfully colonising and expanding asexually in regions where its entire life history may not be expressed (Hu & Juan 2014). Nyberg & Wallentinus (2009) also found that *G. vermiculophylla* can survive 175 days in moist conditions under total darkness, resuming exponential growth following a return to normal light, salinity and immersion conditions. Hence, the species is well suited for long-distance transport in ballast water and on ship hulls or decks and can survive long-term burial in estuarine environments (Nyberg & Wallentinus 2009). Rapid spread of *G. vermiculophylla* around the globe is indicative of broad ecological fitness traits (Nettleton *et al.* 2013), whether through reproductive

versatility or through wide habitat tolerance, allowing success in diverse environments (Hu & Juan 2014).

Gracilaria is a euryhaline genus (Bird *et al.* 1979; McLachlan & Bird 1984; Yu *et al.* 2013). For example, *G. tikvahiae* collected from Tampa Bay, Florida USA, survived salinities between 8 and 60 S and grew well between 15 and 35 S (Bird & McLachlan 1986). Lapointe *et al.* (1984) also found no difference in growth rates of *G. tikvahiae* grown at two different salinities (26 vs 16 S). *Gracilaria vermiculophylla* has an even wider range of tolerances to environmental stresses. Although the optimal salinities for growth of *G. vermiculophylla* are from 15 to 30 S (Rueness 2005), this alga can survive for many weeks at 0.5 S (Weinberger *et al.* 2008) to 60 S (Yokoya *et al.* 1999). *Gracilaria vermiculophylla* also has a wider range of temperature tolerance than *G. tikvahiae*. For example, the optimal growth temperature of *G. vermiculophylla* was from 11°C to 30°C (Yokoya *et al.* 1999; Rueness 2005; Abreu *et al.* 2011), but *G. tikvahiae* did not grow below 15°C (Lapointe *et al.* 1984). *Gracilaria vermiculophylla* survives with suboptimal growth even at temperature extremes as low as 2°C and as high as 35°C (Yokoya *et al.* 1999; Raikar *et al.* 2001; Abreu *et al.* 2011). This suggests that *G. vermiculophylla* will be able to survive fluctuating salinity and temperature environments. For example, recently introduced *G. vermiculophylla* in Long Island Sound (LIS) grows mainly in the subtidal zone and therefore is rarely exposed to hyperosmotic conditions, but it often experiences hypo-osmotic stress due to the input of riverine water. On the other hand, *G. vermiculophylla* growing in shallow embayments and tide pools also

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experiences daily fluctuation of temperature. When the low tide occurs during midday with high/low temperature in summer/winter months, the water temperature could reach values close to the air temperature in shallow embayments and tide pools. Therefore, in estuaries and embayments, *G. vermiculophylla* should be better adapted to short term hypo-osmotic and temperature stresses. Resistance to low-salinity stress or high/low temperatures and rapid recovery from these stresses allow for fast growth and successful invasion into brackish water and embayments.

In addition, global climate change has influenced the invasion of seaweeds. Global climate change warms water temperatures in northern latitudes. An overall warming up to 4.5°C is predicted in the next century as a result of global climate change [Intergovernmental Panel on Climate Change (IPCC) 2014]. Over the past two decades, the water temperatures in Long Island Sound have raised by 0.39°C decade⁻¹ from 1979 to 2002 (though the January–March rate of increase was 0.62°C decade⁻¹; Keser *et al.* 2005). This may cause seasonally stressful conditions for some native species but may provide suitable thermal conditions for nonnative species to thrive in this region. In this case, declines in native species and loss of populations would be the result of the synergistic effects of the global climate change and invasive species (Rahel & Olden 2008). This will enable *G. vermiculophylla* to deal more successfully with global climate change and therefore may become an even more aggressive invader. The objective of this study is to compare the growth and survival strategies under hypo-osmotic (local factor) and temperature (local and global factors) stresses of the native and invasive species of *Gracilaria* in a globally changing environment. Two species of *Gracilaria* [*G. tikvahiae* and *G. cervicornis* (Turner) J. Agardh] native to North America were compared to the invasive *G. vermiculophylla*. These species are similar in terms of growth habits, morphology and frond texture.

MATERIAL AND METHODS

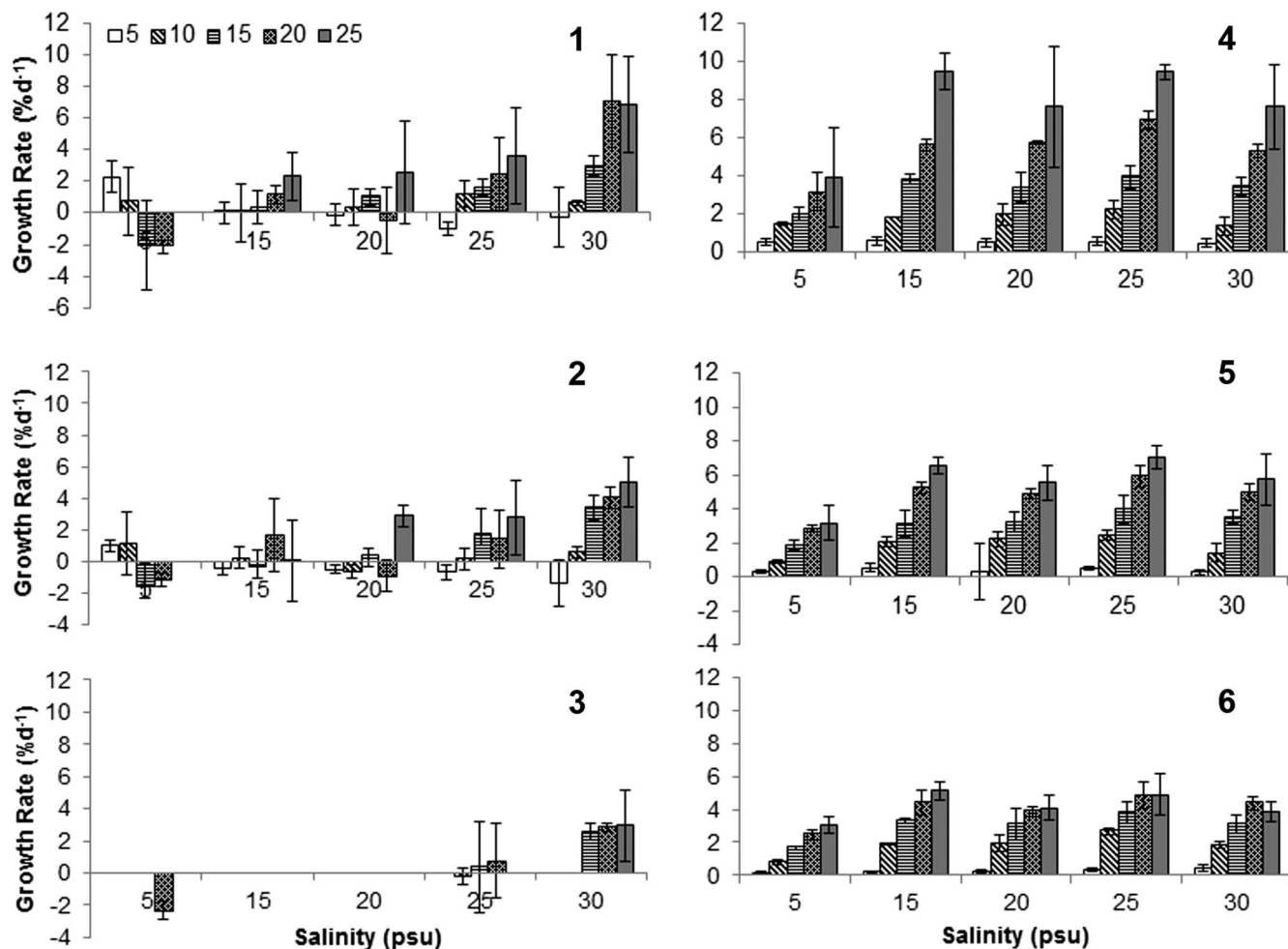
Two local strains of *Gracilaria* were used in this study. The native *G. tikvahiae* (G-RI-ST₁) was originally collected on 18 April 2010 from Potters Pond, South Kingstown, Rhode Island USA (RI; 41°23'45"N, 71°32'13"W). The invasive *G. vermiculophylla* (G-CT₂-ST₁) was collected on 23 April 2010 near the mouth of the Noroton River Estuary, Cove Island, Stamford, Connecticut USA (41°02'57"N, 73°29'56"W). Both strains were isolated and mass cultured in von Stosch's enriched (VSE) seawater at 20°C, S of 28–30, 80–100 μmol photons m⁻² s⁻¹ of photosynthetically active radiation (PAR) and a 12:12 light:dark photoperiod. One-centimeter-long apical segments were obtained from each species. These segments were then acclimated to a standard salinity condition (S = 30), 20°C, 80 μmol photons m⁻² s⁻¹ of PAR and a 12:12 light:dark photoperiod for 7 days to allow for ample recovery from wounding effects of cutting the thalli. The various salinity conditions were made by diluting sterile seawater with deionized water with VSE (Ott 1965).

After 7 days of acclimation, healthy apical segments of each were placed in glass Petri dishes (60 × 10 mm) with 15

ml of VSE medium. Three apical segments were placed in each Petri dish, and each Petri dish was replicated three times. The three replicates of each strain were then placed at each of the five different salinities (S = 5, 15, 20, 25 and 30) in five different growth chambers (Harris Environmental Systems, Andover, Massachusetts USA; Hotpack Corp., Philadelphia, Pennsylvania USA) with different temperatures (5°C, 10°C, 15°C, 20°C and 25°C). The Petri dishes were sealed with Parafilm in order to minimize evaporation and thus control salinity (Yarish & Edwards 1982). Segments were cultivated under their assigned salinity and temperature conditions, a 12:12 light:dark photoperiod and 100 μmol photons m⁻² s⁻¹ for 3 weeks. Culture media were renewed every 3 or 4 days.

Five species/strains of *Gracilaria* were used in this experiment: *G. vermiculophylla* (GV-PT-ST₁) originally collected from Ria de Aveiro, Portugal (40°38'11"N, 8°40'32"W); *G. vermiculophylla* (G-CT₂-ST₁); *Gracilaria tikvahiae* (G-RI-ST₁); *Gracilaria tikvahiae* (GT-CT₁-ST₁), originally collected from the quarry at Millstone Point, Waterford, Connecticut (41°18'21"N, 72°09'40"W); and *Gracilaria cervicornis* (GC-FL-ST₁), originally provided by Thomas R. Capo (University of Miami, Miami, Florida). The temperatures tested ranged from 22°C to 39°C in 2°C or 3°C increments using a temperature gradient table (Yarish *et al.* 1979; Yarish & Edwards 1982). The experiments were conducted using a split-plot, randomized complete block design, with temperature (eight levels) as main plots and species/strain (five levels) as subplots, resulting in a total of 40 treatment combinations. Three segments were cultivated in Petri dishes for 14 days, and each Petri dish was replicated four times. All Petri dishes were photographed at days 0, 1, 3, 6, 9 and 14 using a "PixeLINK" digital camera attached to a dissection microscope. PAR, photoperiod and acclimation conditions were the same as above. The length (hypo-osmotic and temperature experiment) or area (upper lethal temperature experiment) was calculated using the PixeLINK software (μScope Microscopy Software, PixeLINK, Ottawa, Ontario, Canada).

For hypo-osmotic and temperature experiments, a three-way analysis of variance (ANOVA) was used to compare growth rates of *Gracilaria* under varying salinity and temperature conditions. Because a full set of data was not obtained in weeks 2 and 3 due to loss of pigmentation of samples, only the first week's data were compared between species at different temperature and salinity conditions. A repeated measures ANOVA was used for *G. vermiculophylla* since a complete data set was available during the 3-week culture period. Prior to the ANOVA, data were examined for homogeneity of variance. All data sets met this assumption. When the ANOVA indicated a treatment effect of temperature or salinity or an interaction between temperature, salinity and species, Tukey's honestly significant difference analysis ($\alpha = 0.05$) was used as a *post hoc* test to determine pairwise comparison probabilities among treatment levels. A regression was used to determine the connection between growth rate and temperature in each species in week 1. This analysis was conducted using IBM SPSS Statistics 20 (IBM, Armonk, New York USA). For the upper lethal temperature experiment, differences among species and treatments were tested for significance



Figs 1–6. Growth rates of *Gracilaria* grown at different temperature and salinity conditions. Values are presented as $\bar{X} \pm s$ ($n = 3$).

- Fig. 1. *G. tikvahiae* in week 1.
 Fig. 2. *G. tikvahiae* in week 2.
 Fig. 3. *G. tikvahiae* in week 3.
 Fig. 4. *G. vermiculophylla* in week 1.
 Fig. 5. *G. vermiculophylla* in week 2.
 Fig. 6. *G. vermiculophylla* in week 3.

using two-way ANOVA. Multiple *post hoc* comparisons among means were tested by the Student-Newman-Keuls (SNK) test. In all cases, the null hypothesis was rejected at the 5% significance level, according to Sokal and Rohlf (1995).

RESULTS

Effect of hypo-osmotic stress under various temperature conditions

Both salinity and temperature, and a combination of these two factors significantly affected the growth of the two species of *Gracilaria* ($P \leq 0.015$). The native *G. tikvahiae* did not grow or even had negative growth rates at prolonged suboptimal conditions ($S < 20$ and $< 20^\circ\text{C}$). However, the invasive *G.*

vermiculophylla grew equally well across the salinity range of 15–30 (Figs 1–6).

Temperature significantly affected the growth rates in both species. Pooled data sets, regardless of salinity conditions during week 1, showed positive relationships between temperature and the growth rates in both species (*G. tikvahiae*: $F_{1,57} = 26.87$, $P < 0.001$; *G. vermiculophylla*: $F_{1,57} = 433.52$, $P < 0.001$; Fig. 7). The growth rates of both species increased as temperature increased (Fig. 7). The growth rates of *G. vermiculophylla* were significantly higher than that of *G. tikvahiae* ($P < 0.001$). The highest growth rates of *G. tikvahiae* were found at $S = 30$ and 20°C and 25°C in week 1 (6.8% – 7.1% d^{-1}). The highest growth rates of *G. vermiculophylla* were 7.6% – 9.5% d^{-1} at 25°C and $S = 15$ – 30 in week 1. The native *G. tikvahiae* did not survive over 3 weeks of culture at the suboptimal condition ($< 10^\circ\text{C}$ and $S < 20$); whereas, the invasive *G. vermiculophylla* grew continuously at all conditions tested (Figs 1–6).

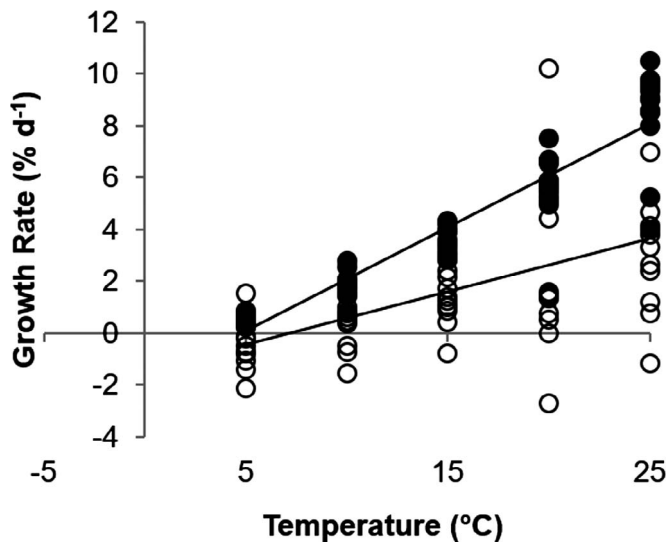


Fig. 7. Growth rate and temperature of *Gracilaria tikvahiae* ($F_{1,57} = 26.87$, $P < 0.001$), open circles, and *G. vermiculophylla* ($F_{1,57} = 433.52$, $P < 0.001$), filled circles.

Determination of upper lethal temperature

In general, the three species of *Gracilaria* tested presented considerable thermal tolerance up to 34°C after 14 days of continuous exposure to these temperatures. All species had survival rates between 0% and 10% after 1 day at the highest temperature tested, 39°C. A similar result was observed with exposure to 36°C, except for *G. vermiculophylla* (CT strain), with several fragments surviving until day 6 (Figs 8–12). At 34°C on day 14, *G. tikvahiae* presented survival rates around 50% and *G. cervicornis* around 75%; whereas, *G. vermiculophylla* presented higher survival (> 80% for the PT strain and 100% for the CT strain; Figs 8–12).

The ANOVA indicated that growth rates were significantly different between species and between temperatures ($P < 0.001$). Strains of *G. vermiculophylla* showed higher growth rates than the other two species for most of the temperatures tested. The CT strain of *G. vermiculophylla*, especially, was able not only to survive at 34°C but also to grow around 10% d⁻¹ (Fig. 13). The growth rates of both strains of *G. vermiculophylla* reached maximum values at 22°C and started to decrease at 34°C ($P < 0.05$). The highest growth rates were observed at 22°C in both strains (14.0% d⁻¹ for the PT strain and 15.4% d⁻¹ for the CT strain; Table 1). The RI strain of *G. tikvahiae* also showed a similar growth pattern but had the highest growth rates at 24°C (8.3% d⁻¹), then dropped to 3.5% d⁻¹. The growth rates of the CT strain of *G. tikvahiae* and of *G. cervicornis* increased as temperature increased up to 31°C and 28°C, respectively (the highest growth rate of 9.2% and 13.1% d⁻¹, respectively), then decreased as the temperature increased further (Fig. 13; Table 1).

DISCUSSION

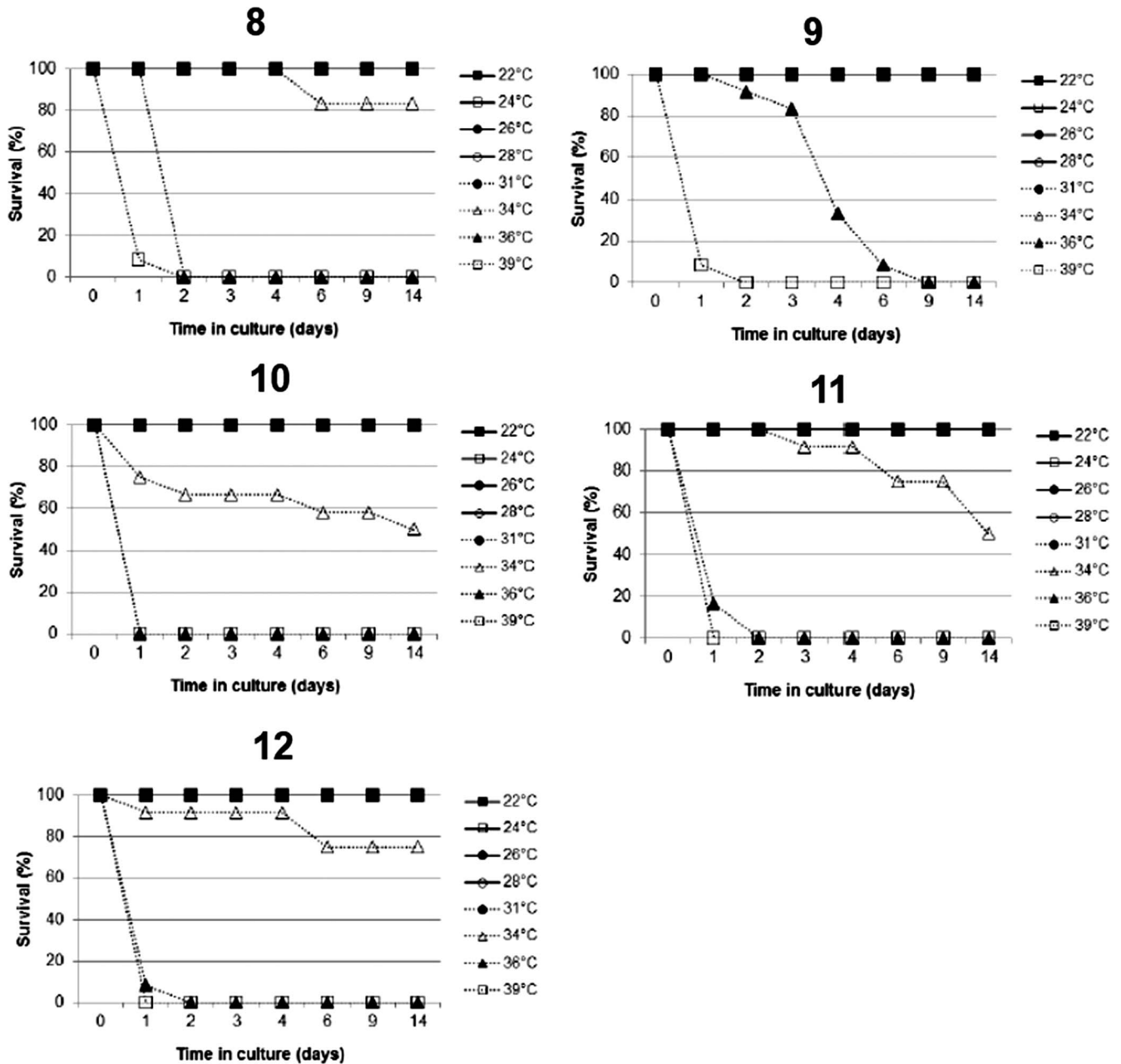
The invasive *G. vermiculophylla* tolerated a wide range of temperature (5°C–34°C) and salinity variations ($S = 5$ –30); whereas, *G. tikvahiae* tolerated harsher environmental

stresses for only a relatively short period of time (2–3 weeks). For example, the native *G. tikvahiae* did not survive prolonged hypo-osmotic ($S \leq 20$) and low- or high-temperature ($\leq 10^\circ\text{C}$ or $\geq 34^\circ\text{C}$) stresses. These differences may help to explain, in part, the success of *G. vermiculophylla* in replacing the native *G. tikvahiae* in the western Atlantic Ocean (South Carolina to New Hampshire; Thomsen *et al.* 2006; Mathieson *et al.* 2008; Nettleton *et al.* 2013) as well as in the eastern Atlantic and the eastern Pacific Oceans (Rueness 2005; Nyberg *et al.* 2009; Gulbransen *et al.* 2012).

In LIS, the average water temperature during winter months is below 5°C, and it even reached 0.4°C in March 2014 (US Geological Survey 2014). The water temperatures in embayments could be even lower during low tides due to their shallow depths. The low temperature along with the salinity fluctuation, especially in embayments, may explain the increase of *G. vermiculophylla* in these habitats and the reduction of native *G. tikvahiae* in LIS (Lopez *et al.* 2014). For instance, *G. vermiculophylla* has mostly (if not completely) replaced the native *G. tikvahiae* in LIS embayments, such as the Noroton River Estuary, Stamford, Connecticut, and Greenwich Cove, Greenwich, Connecticut. The invasive alga was found in these areas even during the coldest winter months when the water temperature was below 1°C (February–March; personal observations). A similar event occurred in Ria de Aveiro Lagoon, Portugal, at somewhat higher temperatures (Lüning 1990). Although it is unclear when *G. vermiculophylla* was introduced to Ria de Aveiro Lagoon (Saunders 2009), this alga has become the dominant macrophyte in this lagoon. Abreu *et al.* (2011) suggested that this successful introduction of *G. vermiculophylla* into Ria de Aveiro Lagoon was probably due to high tolerance of this alga to extreme salinity and temperature fluctuations.

Although *G. tikvahiae* is a euryhaline species (Lapointe *et al.* 1984; Bird & McLachlan 1986), in the present study, most apical segments of *G. tikvahiae* could not survive at $S = 20$ or below for 3 weeks; whereas, *G. vermiculophylla* survived and even grew at the lowest salinity condition tested ($S = 5$) during the same time period. This may be due to the salinity conditions at the original collection sites of each species. *Gracilaria tikvahiae* was collected from Potters Pond, Rhode Island, with an average salinity of 27 (Pfeiffer-Herbert 2007); whereas, *G. vermiculophylla* was collected from the Noroton River Estuary (Stamford, Connecticut USA) with an average salinity of 20 (Yarish & Baille 1989; CH2MHILL 2010). It may also be due to the thalli portions used in these studies. Apical segments were used in the present study; whereas, Bird *et al.* (1979), Lapointe *et al.* (1984), McLachlan & Bird (1984), and Bird & McLachlan (1986) used entire thalli for their studies. Apical segments of *Gracilaria* are known to be more susceptible to stresses, but in our studies, we permitted wound repair of the tissue during the 7 days of acclimation. In other studies with short acclimation times, apical segments of *G. tenuistipitata* lost pigmentation at $S = 3$ within 2 days, and necrosis occurred after 4 days (Chaoyuan *et al.* 1993). In the present study, a complete depigmentation of the apical segments occurred at $S = 15$ or below in *G. tikvahiae* within 3 weeks, regardless of temperature conditions.

Gracilaria vermiculophylla has not only a higher tolerance to stresses but also a higher growth capacity than *G.*



Figs 8–12. Survival rate of the apical fragments of *Gracilaria*, throughout the length of the experiment, grown under different temperatures ($n = 12$).

- Fig. 8.** *G. vermiculophylla*, PT strain.
- Fig. 9.** *G. vermiculophylla*, CT strain.
- Fig. 10.** *G. tikvahiae*, RI strain.
- Fig. 11.** *G. tikvahiae*, CT strain.
- Fig. 12.** *G. cervicornis*, FL strain.

tikvahiae or *G. cervicornis*. This result generally agrees with findings from other studies (Bird & McLachlan 1986; Raikar *et al.* 2001; Abreu *et al.* 2011); although, an exceptional growth of *G. tikvahiae* was reported in New York City’s estuary (Kim *et al.* 2014). In North America, *G. tikvahiae* occurs from the Gulf of Mexico to the southern Gulf of St. Lawrence, and the tropical/subtropical species *G. cervicornis* was found only in Florida, Texas and Mexico (Guiry & Guiry 2015). Although *G. vermiculophylla* has not been

reported in the Gulf of Maine or the Gulf of Mexico, its broader tolerance to temperature and hypo-osmotic stresses will enable this invasive alga to expand its range.

Almost all invasive populations (99%) of *G. vermiculophylla* in Europe and eastern and western North America share a haplotype with populations in its native locations, such as eastern Korea, western Japan and Russia (Kim *et al.* 2010). During invasions in Europe and North America, this alga should have adapted to different local environments. In

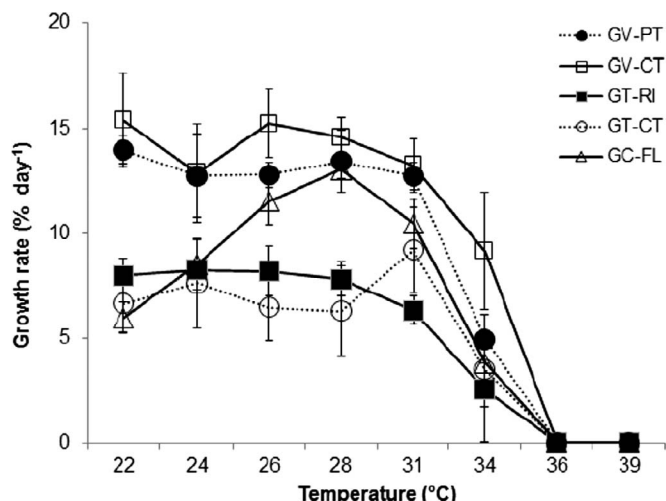


Fig. 13. Average daily growth rate (% d⁻¹) of five strains of *Gracilaria* grown at different temperatures during 14 days (*n* = 4). GV-PT, *G. vermiculophylla* from East Atlantic (Portugal); GV-CT, *G. vermiculophylla* from Connecticut; GT-RI, *G. tikvahiae* from Rhode Island; GT-CT, *G. tikvahiae* from Connecticut; GC-FL, *G. cervicornis* from Florida.

the present study, the Portugal and CT strains responded differently to the thermal stress. At 34°C, the survival of the Portugal strain decreased within 6 days; whereas, the CT strain remained at 100% survival over the entire 14 days of cultivation. Interestingly, a similar physiological feature was also found in the native strains of *G. tikvahiae* tested in the present study. The CT strain of *G. tikvahiae* was originally collected from a quarry adjacent to the Millstone nuclear power plant where the water temperatures are at least a 5°C–10°C higher than LIS year-round (Keser *et al.* 2005). Even during winter months, the water temperature remains above 10°C; whereas, the water temperature in LIS drops below 1°C (Keser *et al.* 2005). This CT strain of *G. tikvahiae* showed a higher survival than the PT strain of *G. vermiculophylla* and grew nearly 10% d⁻¹ even at 31°C. However, for the RI strain collected at Potters Pond, Rhode Island, with no obvious thermal impacts, the survival decreased to approximately 80% within 6 days.

In conclusion, the findings from the present study suggest the broad environmental tolerances of *G. vermiculophylla*, such as broad tolerance to temperature and low salinity, have enabled the expansion of its range into new environ-

ments. Our data explain the successful invasion of this alga into LIS, also along the coasts of the North Atlantic Ocean and elsewhere. The unusual weather events due to, at least in part, global climate changes (IPCC 2014), such as extreme winter, summer or storms, have caused short-term dramatic changes in water temperature and salinity. These unusual weather events may have also caused or accelerated, in part, the recent replacement of the native *G. tikvahiae* by the invasive *G. vermiculophylla* in LIS and other regions. The wider tolerance to temperature in *G. vermiculophylla* will be a great advantage in a globally changing environment.

Since populations of *G. vermiculophylla* on the coasts of Atlantic Ocean are genetically identical to its native population in Korea, Japan and Russia (Kim *et al.* 2010), a comparison of physiological responses to the stresses between these *G. vermiculophylla* populations will be needed to determine if there are any ecotypic adaptations during the colonization and expansion of this species. It is also important to look at the competitive interactions and relationships with herbivores in determining the eventual success of competing species in light of the recent reports of Hammann *et al.* (2016). This recent study reported that the introduced *G. vermiculophylla* in Europe and North America contains much more prostaglandin, a hormone-like toxic compound, than native populations in Asia. This increased toxicity may provide protection against animal consumers. Finally, determination of genetic variations between populations of the native *G. tikvahiae* (thermo-impacted vs nonimpacted) in LIS should also be tested.

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Table 1. Average daily growth rate (% d⁻¹) ± *s* of five strains of *Gracilaria* grown at different temperatures during 14 days (*n* = 4). Results at 36°C and 39°C were not included because all strains died in 2 days except for GV-CT. GV-PT, *G. vermiculophylla* from East Atlantic (Portugal); GV-CT, *G. vermiculophylla* from Connecticut; GT-RI, *G. tikvahiae* from Rhode Island; GT-CT, *G. tikvahiae* from Connecticut; GC-FL, *G. cervicornis* from Florida. In each row, different lowercase letters denote significant differences between the strains for that temperature, according to the SNK test (*P* < 0.05).

Temperature (°C)	Strains: growth rate (% day ⁻¹) ± <i>s</i>				
	GV-PT	GV-CT	GT-RI	GT-CT	GC-FL
22	14.0 ± 0.32 a	15.4 ± 1.13 a	8.0 ± 0.40 b	6.7 ± 0.68 b	6.0 ± 0.38 b
24	12.7 ± 1.00 a	12.9 ± 1.18 a	8.3 ± 0.24 b	7.6 ± 1.08 b	8.5 ± 0.60 b
26	12.8 ± 0.30 b	15.3 ± 0.82 a	8.2 ± 0.59 c	6.5 ± 0.80 c	11.6 ± 0.57 b
28	13.5 ± 0.75 a	14.60 ± 0.46 a	7.8 ± 0.40 b	6.3 ± 1.07 b	13.1 ± 0.75 a
31	12.8 ± 0.31 a	13.3 ± 0.65 a	6.3 ± 0.35 c	9.2 ± 1.01 b	10.5 ± 0.59 b
34	5.0 ± 0.58 b	9.2 ± 1.40 a	3.7 ± 0.54 b	3.5 ± 0.88 b	3.8 ± 0.45 b

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