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Ecophysiological studies of the non-indigenous species *Gracilaria vermiculophylla* (Rhodophyta) and its abundance patterns in Ria de Aveiro lagoon, Portugal

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Gracilaria vermiculophylla is the dominant macrophyte in the Ria de Aveiro lagoon, Portugal (40°38'N, 8°43'W), which is a highly urbanized estuary impacted by oyster cultivation and finfish aquaculture. This study aimed to understand the success of *G. vermiculophylla* in estuaries by monitoring its abundance and phenology throughout an annual cycle. This species thrives in soft-bottom communities, mostly entangled in the substrate amongst dead bivalve shells and tubes of the polychaete *Diopatra neopolitana* but it may be also found free-floating; it is present year-round. Carposporophytes were found throughout the year and at all sampling locations. The life history of this taxon was completed in the laboratory. Spore germination and growth success were investigated in a fully factorial designed array of temperatures (5, 10, 15, 20 and 25°C), photoperiods (8, 12 and 16 h day-lengths) and photon flux densities (40 and 100 µmol photons m⁻² s⁻¹). Germination occurred under all conditions, being highest at 20°C under the long day treatment. Germination was lowest at 5°C, with no spore survival. The growth rates of the sporelings were affected by the interaction of the three factors tested. These were minimal at 10°C (2.21% ± 1.14 in 8-h days for tetrasporophytes and 2.60% ± 0.57 in 12-h days for gametophytes) and maximal at 20°C and long day treatments for both gametophyte and tetrasporophyte phases (9.96% ± 0.62 and 16.86% ± 0.73, respectively). No differences were found in the growth rates of the mature haploid or diploid phases. *Gracilaria vermiculophylla* is reproductive throughout the year. It attains high rates of germination and growth success under a wide range of environmental conditions. With such broad tolerances to environmental factors, *G. vermiculophylla* has a high invasive potential, which helps to explain its current dominance in the Ria de Aveiro lagoon.

Key words: ecophysiology, growth rate, *Gracilaria vermiculophylla*, life cycle, non-indigenous species, Rhodophyta, seaweed, spore germination, temperature

Introduction

Non-indigenous species (NIS) are those (1) occupying niches in areas where they were not previously present, (2) geographically separate from their native range and (3) normally transported by means of anthropogenic vectors (Boudouresque & Verlaque, 2002). These species can be more efficient than the native ones in using available resources or in their ability to respond to disturbance (Occhipinti-Ambrogi & Savini, 2003). Non-indigenous species are often perceived as having negative effects, eventually leading to the extinction of native species. Nevertheless, NIS may also have neutral or positive effects in the community by creating new

niches and increasing local biodiversity (Williams & Smith, 2007; Boudouresque, 2008). After NIS establishment, there are several management alternatives (Bax *et al.*, 2001). If they have economic value, for instance, NIS can be managed as a new exploitable resource (Inderjit *et al.*, 2006).

Gracilaria vermiculophylla is a NIS from north-east Asia (Kim *et al.*, 2010). It was first reported in European waters, including a Portuguese location (Ria Formosa, 36°59'N, 7°55'W), by Rueness (2005). The most abundant *Gracilaria* species thriving at Ria de Aveiro lagoon (40°38'N, 8°43'W) was previously referred to as *Gracilaria verrucosa* (Silva, 1985; Silva *et al.*, 2004) or *G. bursa-pastoris* (Matos *et al.*, 2006; Araújo *et al.*, 2009). Saunders (2009) confirmed the existence of *G. vermiculophylla*, *G. gracilis* and *Gracilariopsis longissima* in

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the lagoon. The exact time of introduction of *G. vermiculophylla* at Ria de Aveiro lagoon is thus still unclear. The first possible report of this species may have been an 1848 record of *G. verrucosa* (Ardré, 1970); however, this can be confirmed only by molecular identification of these old herbarium specimens. Later, in local commercial reports, it is referred to as a component of a mixture of seaweed and seagrasses ('moliço') used as a soil conditioner, in which it was combined with *Potamogeton pectinatus*, *Ruppia cirrhosa*, *Lamprothamnium papulosum* and *Zostera noltii* (Silva 1985). Currently, *G. vermiculophylla* is the dominant macrophyte in the Ria de Aveiro lagoon, together with *Z. noltii* and *Ulva* species (Silva *et al.*, 2004). The mud and fine sand flats that characterize this sheltered soft-bottom lagoon offer an ideal site for the establishment of this species (Terada & Yamamoto, 2002).

G. vermiculophylla is able to propagate via fragmentation, and is both euryhaline and eurythermal (Yokoya *et al.*, 1999; Rueness 2005). It is able to survive long periods of darkness (Nyberg & Wallentinus, 2009) and it is resistant to desiccation and herbivory (Thomsen & McGlathery, 2007). All these traits make *G. vermiculophylla* a very successful colonizer in estuarine environments. Recent studies on the north-east coast of the Pacific (Bellorin *et al.*, 2004; Piñon-Gimate *et al.*, 2008) and in the Baltic Sea (Nyberg *et al.*, 2009) have shown that this species establishes itself and spreads rapidly.

The ecological and physiological traits of a NIS, together with features of the recipient area, will determine its colonizing success (Nyberg & Wallentinus, 2005). Temperature is a primary environmental factor regulating the germination and growth of macroalgae (Yarish *et al.*, 1984, 1986; Egan *et al.*, 1989; Lüning, 1990). Nevertheless, photoperiod and irradiance levels are also fundamental for their development (Yarish *et al.*, 1984; Lüning, 1990). Previous studies have described some physiological traits of *G. vermiculophylla* (Yokoya *et al.*, 1999, Raikar *et al.*, 2001, Rueness 2005). However, the multi-factorial effects of the environment on *G. vermiculophylla* physiology have been evaluated only in field-based experiments (Thomsen & McGlathery, 2007, Thomsen *et al.*, 2009). Hence, ours is the first comprehensive study on the influence of temperature, photoperiod and photon flux density on spore germination and growth of the haploid and diploid phases of *G. vermiculophylla*, under laboratory controlled conditions. We also aimed to understand the generally high success of *G. vermiculophylla* in estuaries by monitoring its abundance and phenology in a lagoon where it is dominant.

Materials and methods

Study site

The Ria de Aveiro, located on the west coast of Portugal (Fig. 1), is a shallow, bar-built or lagoon-type estuary that is permanently connected to the ocean by a single opening. It is about 45 km long (NNE–SSW) and 8.5 km wide. The mean depth of the lagoon is about 1 m (the tidal range being 1 to 3 m), except in the navigation channels, where dredging operations maintain a depth of about 7 m. In the area where the study was performed, the monthly mean values of water temperature and salinity range from 12 to 23°C and 15 to 36 ppm, respectively (Lopes *et al.*, 2007).

Preliminary survey

An initial survey was done on the intertidal of the main channels of the Ria de Aveiro lagoon (Fig. 1). Macrophytes were identified and their positions marked by GPS. A *Gracilaria* species was confirmed as the dominant macrophyte, in accordance with Silva *et al.* (2004) (misidentified then as *Gracilaria verrucosa*). To confirm the taxonomic position of all the Gracilariales thriving in the lagoon, samples of all the morphotypes were collected, preserved in silica gel and sent to Dr Gary Saunders' laboratory for molecular



Fig. 1. Geographical position of the Ria de Aveiro Lagoon on the western coast of Portugal and location of the study area (black rectangle).

analysis (Saunders, 2009). *Gracilaria vermiculophylla*, *G. gracilis* and *Gracilariopsis longissima* were positively identified (see Introduction). In the field, *Gracilaria vermiculophylla* can be distinguished by its dark red to black colour and high degree of ramification. Most of the *G. vermiculophylla* patches in the lagoon were difficult or impossible to access. So, within the constraint of safe access for sampling, three *Gracilaria* patches (400 m to 1.2 km apart) were haphazardly selected for this study (A: 40°38'2.63"N, 8°40'33.05"W; B: 40°38'11.28"N, 8°40'3.18"W; C: 40°38'20.67"N, 8°39'50.41"W).

Abundance patterns

From September 2006 to July 2007, we monitored the abundance and reproductive status of the three *G. vermiculophylla* patches in the Ria de Aveiro. At the beginning of the study we placed four marks at the edges of each patch and measured the distances to fixed points on the shore, as well as between the marks; initial areas ranged from 111 to 205 m². Two digital photos per patch were taken every 2 months, always from the same positions. The variation of the area of the patches was analysed through the web freeware program ImageTool3.0 (<http://ddsdx.uthscsa.edu/ImageTool.asp>). Preliminary trials, to assess the best way to estimate the abundance of algae in these areas, showed that the relationship between percentage cover and biomass was not linear. Therefore, we decided to combine digital photography with destructive biomass estimates from five randomly chosen 0.25 m² squares with 100% cover of *G. vermiculophylla*. In order to guarantee time-independent data, care was taken to sample different quadrats at each sampling date (numbered grid + random number table).

Phenological observations

Gracilaria vermiculophylla exhibits a *Polysiphonia*-like life history with two independent isomorphic reproductive phases (Ohmi, 1956). To study the reproductive seasonal dynamics of *G. vermiculophylla*, 50 'individuals' were sampled randomly in the laboratory from the biomass collected in each patch and their reproductive status was determined for all sampling dates. After cleaning the plants, carposporophytic material was easily identified by the naked eye. For the identification of mature male gametophytes and tetrasporophytes, a dissection or optical microscope was used for detailed observations. In the case of vegetative plants, tetrasporophytes and gametophytes could not be distinguished morphologically.

Life-history studies

Carposporophytic material of *G. vermiculophylla* was collected from the populations in the lagoon. Several small portions of thalli bearing only one cystocarp were cleaned with sterilized seawater; each portion was individually placed in a 250 ml Petri dish containing von Stosch Enrichment (VSE) solution (Ott, 1965) with GeO₂ solution (at 4 mg l⁻¹, to prevent proliferation of diatoms). Dishes were left overnight at room

temperature for spore liberation (~10 h). Spores were isolated with the aid of a modified Pasteur pipette (see Pereira *et al.*, 2004) from the Petri dishes with the highest concentrations of spores and distributed among slides (100–150 spores counted per slide). The slides with the isolated spores were placed inside a 'moisture chamber' (a tightly closed darkened plastic container with distilled water beneath a rectangular glass plate elevated by 1 cm deep Petri dishes) and kept inside a 15°C incubator; this helps spores adhere to glass slides and prevents cell division.

Germination rates

The effects of temperature, photon flux density (PFD) and photoperiod on the germination rates of *G. vermiculophylla* carpospores and tetraspores were evaluated. The full factorial design included three temperatures (10, 15 and 20°C), three photoperiods S (8 h day-length), N (12 h day-length) and L (16 h day-length) and two PFDs (40 and 100 µmol photons m⁻² s⁻¹). In order to include more extreme temperatures, the effects of 5 and 25°C, combined with the same PFD levels, were tested on the germination rates under a 12:12, L:D (=day-neutral photoperiod), for a total of 22 experimental treatments. The notation adopted for each treatment includes the level of each factor being tested (e.g. 10N40 = 10°C, 12 h day-length and 40 µmol photons m⁻² s⁻¹). The work was done in growth chambers programmed for each set of temperatures and photoperiods tested (11 chambers in total). The different light intensities were achieved by shading inside the growth chambers.

The day immediately after spore isolation, initial observations (T₀) were made on all the slides. Slides with fewer than 100 settled spores were discarded. The number of spores germinating within the first 30 spores observed (at random) was counted to check for any initial germination. The slides were then placed individually in Petri dishes filled with VSE, which were randomly distributed among the different treatments; there were six Petri dish replicates for carpospores and four replicates for tetraspores. After four days under the experimental conditions, 30 spores per slide (giving a total of 180 carpospores and 120 tetraspores per treatment) were examined under an inverted microscope and the percentage of germinated spores assessed. Germination was considered positive when two or more cell divisions were observed (Yarish *et al.*, 1979, 1982).

Growth rates – young sporelings

After the fourth day, spores incubated at 5°C showed very low survival rates, so we decided to exclude this treatment for the growth experiment. All other levels of the experimental design were kept exactly as described in the germination rate experiment. At the start of the experiment, the sporelings were *c.* 30 days old (T₀ = day of settlement) and had average thallus lengths of 9 mm and 4 mm, for young tetrasporophytes and gametophytes respectively. Sporelings were grown



Fig. 2. *Gracilaria vermiculophylla* in the Ria de Aveiro, Portugal (40°38'20.67"N, 8°39'50.41"W); photograph taken in November 2006.

inside deep Petri dishes (three replicates for tetrasporophytes and four replicates for gametophytes) with 250 ml of VSE culture media, which was renewed weekly. Since weight changes were impossible to determine accurately at this early stage of development (small weights and high variation due to drying times), we chose to calculate the Relative Growth Rate (RGR) of the sporelings based upon the length of the central axis of the thalli (side-branching was not considered) over time (15 days):

$$RGR = \frac{\ln FL - \ln IL}{t} \times 100$$

where *FL* is the final length, *IL* is initial length and *t* is the time (in days) between measurements.

Growth rates – mature tetrasporophytes and gametophytes

An experiment was designed to compare the growth performance of mature female, male and tetrasporophyte individuals (initial fresh weight ~2 g). With clonal cultures obtained from apical cuttings of the sporelings with the best growth performance, a factorial design was applied, having two different strains (i.e. originating from two sporelings) (S) for each reproductive status (R) and two replicates per R(S) combination. The experiment was done at 20°C, day-neutral photoperiod and a PFD of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Cultures were kept in VSE and the culture media was renewed every 3–4 days during the 3 weeks of the experiment. The seaweed density in 1 L flasks was maintained at 2 g (FW) l^{-1} by weekly removal of excess biomass. The RGR of each replicate was calculated using the same formula as above but measuring fresh weight instead of length.

Data analysis

Analysis of variance (ANOVA) was performed on the germination of carpospores and tetraspores and on the growth rates of young tetrasporophytes and

gametophytes, with temperature, photoperiod and PFD as factors (Tables 2 and 3). Prior to analysis, data were tested for variance heterogeneity with Cochran's Test and, when necessary, an arcsine transformation was applied (Underwood, 1997). Significant differences found were identified *a posteriori* with Student–Newman–Keuls (SNK) tests, using the software package GMav v5 (EICC, University of Sydney, Australia).

Results

Abundance patterns

The preliminary survey revealed that *G. vermiculophylla* is abundant throughout the lagoon. It occurs in dense homogeneous patches (Fig. 2), sometimes including other macroalgal species (*Ulva* spp., *Fucus* sp.) or the seagrass, *Zostera noltii*. *Gracilaria vermiculophylla* thrives mostly entangled in the soft-bottom muddy substrate, attached to dead bivalve shells or to the tubes of the polychaete, *Diopatra neopolitana*. It can also be seen free-floating, entangled on the culms of the salt-marsh grasses, or washed onto the margins of the lagoon.

The patches of *G. vermiculophylla* chosen for this study proved to be ephemeral. It was impossible to perform a rigorous statistical analysis of the data obtained and the observations are therefore only qualitative. Data on the variation of the area of the sampled patches and the corresponding biomass per unit area are given in Table 1. Despite the similarity of the patches on the first sampling date, only one of the patches persisted throughout the study, its total area ranging from 204 m^2 (September) to 2101 m^2 (July). However, although the biggest area was registered in July, it corresponded to a minimum in biomass ($1.23 \pm 0.15 \text{ kg (FW) m}^{-2}$). Maximal abundance

Table 1. Abundance data obtained from the *G. vermiculophylla* patches. **A:** 40°38'2.63"N, 8°40'33.05"W, **B:** 40°38'11.28"N, 8°40'3.18"W and **C:** 40°38'20.67"N, 8°39'50.41"W. Area units = m² and biomass units = kg (FW) m⁻².

Sampling month	A		B		C	
	Area	Biomass	Area	Biomass	Area	Biomass
September	204	2.26 ± 0.11	122	1.90 ± 0.17	111	2.18 ± 0.21
November	1478	1.47 ± 0.10	441	2.54 ± 0.43	299	3.57 ± 0.13
January	485	2.27 ± 0.40	391	1.94 ± 0.12	0	0
March	271	1.48 ± 0.09	24	1.78 ± 0.11	0	0
May	1890	2.19 ± 0.24	55	2.10 ± 0.15	0	0
July	2101	1.23 ± 0.15	0	0	0	0

was measured in January (2.27 ± 0.40 kg (FW) m⁻²).

Phenological observations

Reproductive individuals of *Gracilaria vermiculophylla* were found year-round. Although the biomass was not sufficient to establish reliable abundance estimates, we could always find at least 50 individuals in all patches. There were large differences between the three patches (Fig. 3). The material collected from patch A was nearly 100% reproductive, except in January (c. 40%). The other two sites followed a very different pattern, with the highest number of reproductive individuals in January. Observations were made of cystocarpic and tetrasporic ramets emerging from the same tuft of *Gracilaria* axes. Due to the difficulty in accurately determining the tetrasporophyte:gametophyte ratio, the results expressed in Fig. 3 refer only to reproductive vs non-reproductive material. During the entire course of the study, carposporophytic material was the most abundant, with cystocarps densely distributed over the whole thallus. Tetrasporophytes were also frequent but mature male thalli were never found.

Life-history studies and germination rates of spores

Temperature × Photoperiod × PFD. Germination of carpospores and tetraspores occurred over the entire range of conditions tested (Fig. 4). Nevertheless, the combined temperature, photoperiod and PFD conditions had different effects on the two types of spores (Table 2).

Germination rates of carpospores were significantly affected by the interaction between temperature and photoperiod ($P < 0.05$, Table 2) and were higher with increasing temperature and photoperiod (Fig. 4A-1). No differences were detected for the two levels of PFD tested. At lower temperatures, significant differences were found

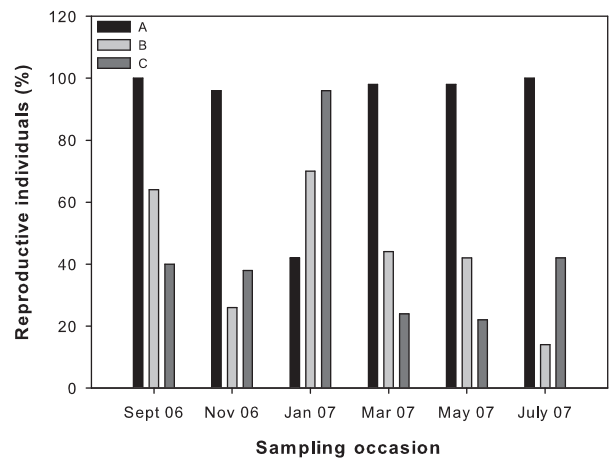


Fig. 3. Percentages of *Gracilaria vermiculophylla* reproductive individuals (as fertilized females + tetrasporophytes) observed at sites A, B and C throughout the year ($n = 50$).

between the different photoperiod conditions (8 h < 12 h < 16 h). With the increase in temperature, despite higher germination in 16 h day-lengths, these differences were not significant. The lowest germination rate (c. 59%) was at 10°C and short day and the highest (c. 99%) under 20°C and long day conditions.

The differences observed in the germination rates of tetraspores were caused by the interaction between the three factors ($P < 0.05$, Table 2). As seen in Fig. 4A-2, germination rates were near 100% for the majority of the conditions tested. Significant differences were solely due to the results obtained under 10N40 and 15L40, with minimum values observed in the 15L40 treatment (75.4% ± 4.48).

Temperature × PFD. The experiment under day-neutral conditions showed that both carpospores and tetraspores germinated at the extreme temperatures tested, i.e. 5 and 25°C (Fig. 4B). The lowest germination rates were observed at 5°C and 40 μmol photons m⁻² s⁻¹ with 23.9% ± 6.41 and 33.3% ± 7.82 for carpospores and tetraspores, respectively. Despite the germination observed under these conditions, the germlings eventually

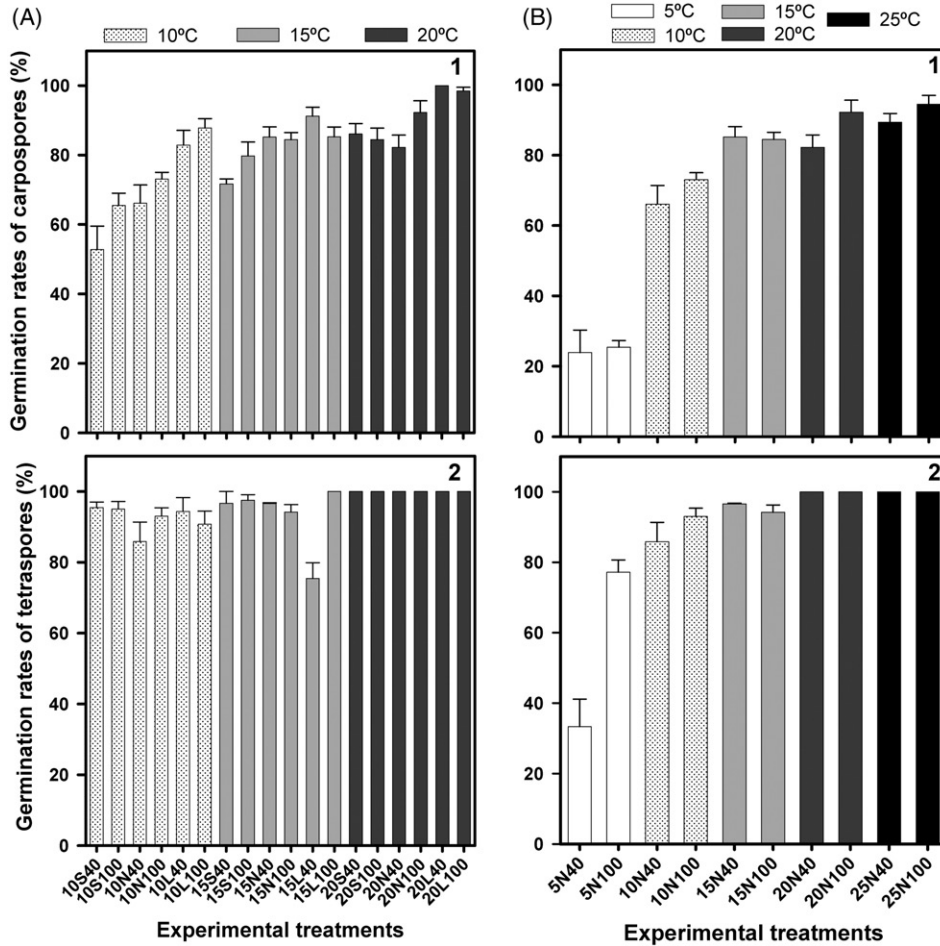


Fig. 4. Germination rates of (1) carpospores (mean \pm standard error, $n = 6$) and (2) tetraspores (mean \pm standard error, $n = 4$) after four days under: (A) different temperature (10, 15 and 20°C), photoperiod (S, N and L) and irradiance (40 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) regimes; (B) different temperature (5, 10, 15, 20 and 25°C) and irradiance (40 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) regimes in a day-neutral photoperiod (N).

Table 2. Analysis of variance examining the effects of temperature (T), photoperiod (PP) and photon flux density (PFD) on the germination rates of carpospores and tetraspores of *Gracilaria vermiculophylla*. *indicates significant differences found between treatments ($P < 0.05$); NS, not significant; df, degrees of freedom; MS, mean-square.

Source of variation	Carpospores						Tetraspores					
	T \times PP \times PFD			T \times PFD			T \times PP \times PFD			T \times PFD		
	df	MS	F	df	MS	F	df	MS	F	df	MS	F
T	2	2639.5	59.9*	4	4162.4	74.8*	2	415.0	16.9*	4	2358.7	69.5*
PP	2	2437.0	55.3*				2	99.8	4.1*			
PFD	1	116.5	2.6	1	296.6	5.3*	1	153.1	6.2*	1	435.3	12.8*
T \times PP	4	145.8	3.3*				4	85.5	3.5*			
T \times PFD	2	71.6	1.6	4	56.6	1.0	2	100.0	4.1*	4	288.2	8.5*
PP \times PFD	2	130.9	2.9				2	80.8	3.3*			
T \times PP \times PFD	4	90.1	2.0				4	214.1	8.7*			
Residuals	90	44.1		50	55.6		54	24.6		30	33.9	
Cochran's Test	C = 0.1200, NS			C = 0.2115, NS			C = 0.2671, NS			C = 0.2729, NS		
Transformation	ArcSin(%)			ArcSin(%)			None			ArcSin(%)		

died; this was assessed by daily observations after the fourth day of the germination experiments. Maximal values of germination rate for both types of spores were observed at 20 and 25°C.

Germination of carpospores was significantly affected by temperature and PFD ($P < 0.05$, Table 2). Germination increased with increasing temperature (up to 20°C) and was greater at

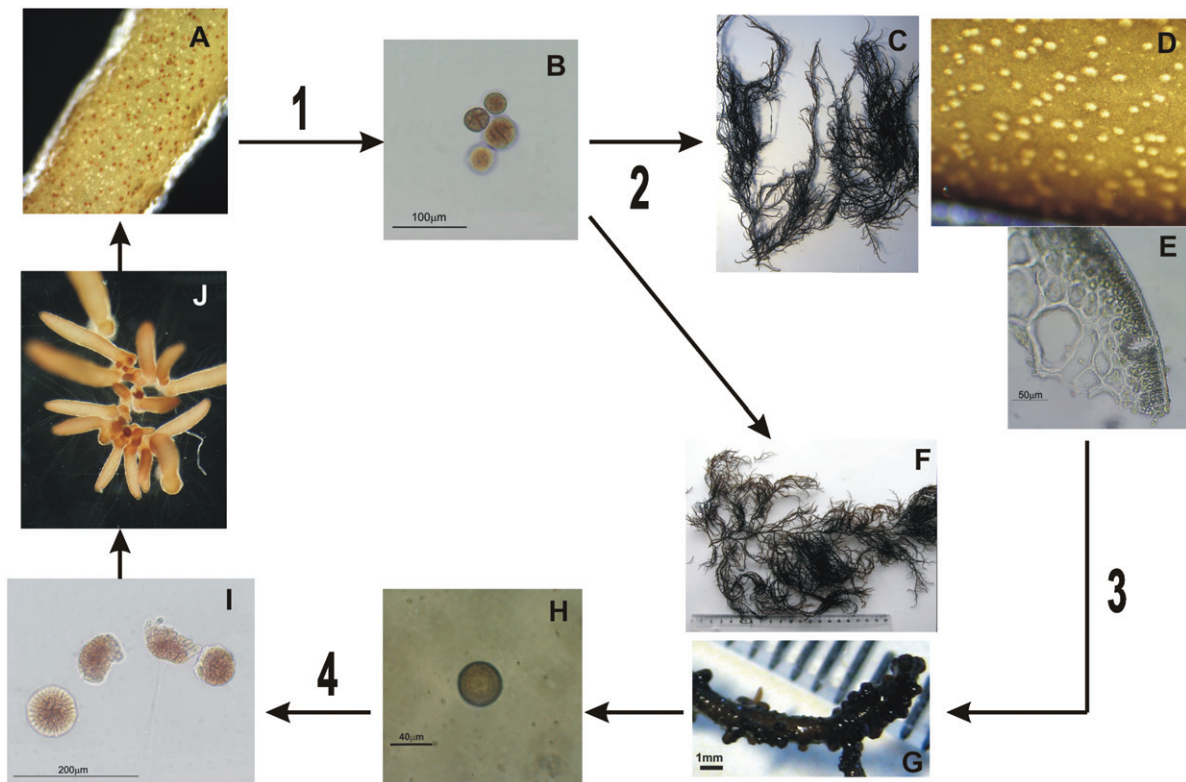


Fig. 5. The life cycle of *Gracilaria vermiculophylla*. **A.** Tetrasporophyte (2n) with tetrasporangia. **B.** Tetraspores (n). **C.** Male gametophyte (n) with spermatangial sori (**D**) and ‘verrucosa’ type spermatangia (**E**). **F.** Female gametophyte (n). **G.** Carposporophyte (2n) with cystocarps. **H.** Carpospores (2n). **I.** Germinated carpospores. **J.** Carposporelings (2n). **1.** Meiosis. **2.** Germination of tetraspores. **3.** Fertilization. **4.** Germination of carpospores.

100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under all temperatures tested, except at 15°C (Fig. 4B-1).

For tetraspore germination, there were no clear differences between temperatures. Interaction between PFD and temperature had a significant effect on germination ($P < 0.05$, Table 2), although this was solely due to the difference between spores germinating under low or high PFD at 5°C (Fig. 4B-2). Development of tetraspores was faster at higher temperatures. In all conditions tested, sporeling coalescence was observed.

Growth rates: young sporelings

All the steps of the life history of *Gracilaria vermiculophylla* were completed in the laboratory (Fig. 5). Selected strains of gametophytes and tetrasporophytes are being kept in culture for possible future studies.

The interaction between temperature, photoperiod and PFD had a significant effect on the daily RGR of the tetrasporophytes and gametophytes ($P < 0.05$, Table 3), as can be seen in Fig. 6A. The RGR of the tetrasporophytes reached average minimum values ($2.2\% \text{ day}^{-1} \pm 1.14$) in the 10S100 treatment, and maximum values of $10.0\% \text{ day}^{-1} \pm 0.62$ in 20L100 conditions (Fig. 6A-1). Under short days and low PFD conditions, there was no significant difference among temperatures.

With increasing temperatures and PFD, the significant difference between photoperiods disappeared. Nonetheless, it was possible to observe a pattern of higher RGR under long day-lengths (Fig. 6A-1).

The RGR of the gametophytes was higher with increasing temperatures (Fig. 6A-2). The SNK tests showed that, in short days, there was a clear difference between 10, 15 and 20°C. On the other hand, with day-lengths of 12 h and 16 h, gametophytes performed in the same way at 15 and 20°C. RGR was always higher under high PFD ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), except for the long-day conditions. The highest daily RGR ($16.9\% \text{ day}^{-1} \pm 0.73$) was observed at 20°C with long days and $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Gametophytes grew less under 10°C, with the minimum RGR ($2.6\% \text{ day}^{-1} \pm 0.57$) at 10N100.

At 25°C, the interaction between the two factors tested (temperature \times PFD) was significant for both tetrasporophytes and gametophytes growth ($P < 0.05$, Table 3). Under $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, the RGR of the tetrasporophytes was significantly different between three groups of temperatures ($10^\circ\text{C} < 25^\circ\text{C} = 15^\circ\text{C} < 20^\circ\text{C}$), with a maximum value of $9.5 \pm 0.25\% \text{ day}^{-1}$ (Fig. 6B-2). On the other hand, at $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, temperature did not make a difference to the growth rates of the tetrasporophytes.

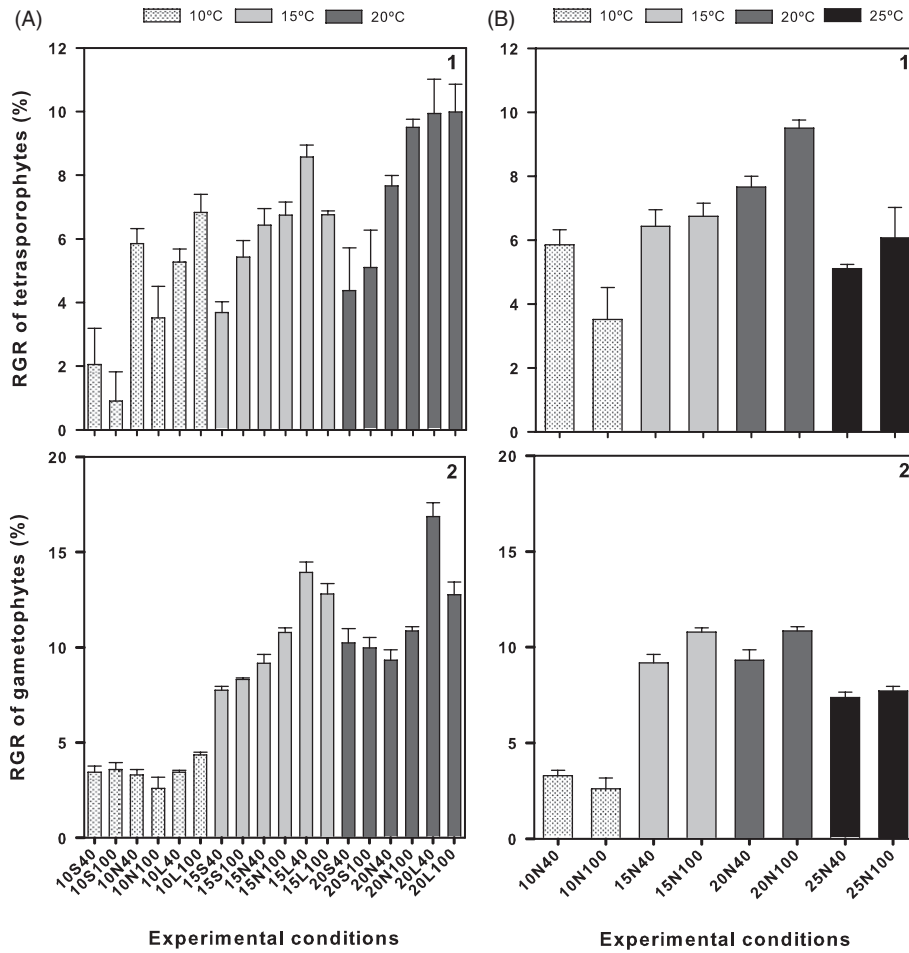


Fig. 6. Growth rates of (1) young tetrasporophytes (mean ± standard error, $n=3$) and (2) gametophytes (mean ± standard error, $n=4$) under: (A) different temperature (10, 15 and 20°C), photoperiod (S, N and L) and irradiance (40 and 100 μmol m⁻² s⁻¹) regimes; (B) different temperature (10, 15, 20 and 25°C) and irradiance (40 and 100 μmol m⁻² s⁻¹) regimes in a day-neutral photoperiod (N).

In turn, the RGR of the gametophyte was significantly higher at 15 and 20°C, under a PFD of 100 μmol photons m⁻² s⁻¹ ($10.8 \pm 0.22\%$ day⁻¹), as well as under 40 μmol photons m⁻² s⁻¹ ($9.3 \pm 0.54\%$ day⁻¹) (Fig. 6B-2).

Young tetrasporophytes became reproductive approximately 2 months after germination in the 15L40, 15L100, 20N100, 20L40 and 20L100 treatments. In other words, tetrasporophytes became reproductive in 16-h days at 15 and 20°C and in 12-h days only at 20°C. In all other experimental conditions, *Gracilaria vermiculophylla* did not mature within five months after germination, despite the fact that the individuals grew to sizes similar to those observed in other conditions. The male gametophytes kept at 20 and 25°C, in 12:12 h and 16:8 h L:D photoperiods and at both light levels, became mature after less than 2 months.

Growth rates: mature tetrasporophytes and gametophytes

Under the experimental conditions used, no significant differences were found in the RGR of mature

males, females or tetrasporophytes ($F_{(2,9)}=4.42$, $P < 0.05$). This analysis was performed after verifying that there were no differences among strains ($F_{(3,6)}=2.22$, $P < 0.01$). The mean RGR value for all plants was $8.66 \pm 0.44\%$ day⁻¹.

Discussion

Field studies

Gracilaria vermiculophylla thrives in the Ria de Aveiro lagoon in unattached populations, mostly entangled in the muddy substrate. This is typical for many *Gracilaria* species occurring in estuaries and other sheltered environments (Norton & Mathieson, 1983, and references therein). *Gracilaria vermiculophylla* in the Ria de Aveiro lagoon is distributed in patches, most of them inaccessible by foot and only visible during low-tide periods. After an intensive survey of the lagoon, the patches chosen for monitoring during this study appeared to be stable. However, these patches were in fact ephemeral and two of them virtually disappeared during the study period.

Table 3. Analysis of variance examining the effects of temperature, photoperiod and photon flux density (PFD) on the growth rates of young tetrasporophytes and gametophytes of *Gracilaria vermiculophylla*. *indicates significant differences found between treatments ($P < 0.05$); NS, not significant; df, degrees of freedom; MS, mean-square.

Source of variation	Tetrasporophytes						Gametophytes					
	T × PP × PFD			T × PFD			T × PP × PFD			T × PFD		
	df	MS	F	df	MS	F	df	MS	F	df	MS	F
T	2	62.0	36.9*	3	16.8	16.3*	2	471.8	563.9*	3	89.2	155.2*
PP	2	87.9	52.3*				2	85.9	102.7*			
PFD	1	0.2	0.1	1	0.2	0.2	1	0.4	0.5	1	3.9	6.8*
T × PP	4	2.0	1.2				4	16.9	20.2*			
T × PFD	2	2.5	1.5	3	4.9	4.7*	2	2.8	3.4*	3	2.4	4.2*
PP × PFD	2	0.4	0.2				2	8.1	9.6*			
T × PP × PFD	4	5.8	3.5*				4	6.9	8.2*			
Residuals	36	1.7		16	1.0		54	0.8		24	0.6	
Cochran's Test	C=0.1793, NS			C=0.3638, NS			C=0.1552, NS			C=0.2848, NS		
Transformation	None			None			None			None		

This was not accompanied by any visible decrease in *Gracilaria* abundance, as new patches were observed in nearby areas. Aerial photography, coupled with local sampling, would be a way to obtain more accurate information on this species' distribution and abundance in the lagoon. Despite the methodological constraints, the abundance pattern observed in Fig. 3 resembles the pattern of this species in a similar lagoon in Virginia, USA (Thomsen *et al.*, 2006).

The instability of the *Gracilaria* mats might be due to the frequent sediment displacements occurring in the Ria de Aveiro as a result of dredging activities (see Silva *et al.*, 2004), and also to the naturally strong spring tides. These, together with light- or nutrient-limitation of individuals lying under the mat of seaweeds, could lead to the fragmentation of the thalli and consequent instability of the population (Norton & Mathieson, 1983). Another possible cause is interaction with the local fauna. Although association with the polychaete *Diopatra* has been reported to facilitate the establishment of *G. vermiculophylla* (Thomsen *et al.*, 2005), this does not seem to be the case in the Ria de Aveiro lagoon. Casual observations revealed different abundances of the snail *Hydrobia ulvae* between the sites and positive associations of *G. vermiculophylla* with *Hydrobia* and *Littorina littorea* have been reported for this lagoon by Barroso *et al.* (2000) and in northern Europe by Thomsen *et al.* (2007). The different attachment mechanisms of *G. vermiculophylla* to these different substrates (the tubes of *Diopatra* and snail shells) have been found to affect the consistency of the thalli (Thomsen, 2004).

Nyberg *et al.* (2009) reported that *G. vermiculophylla* increased local diversity, which is especially important in non-vegetated mudflats. During this study, besides *Hydrobia ulvae* and *Littorina*

littorea, hundreds of crab recruits were observed to shelter in the *Gracilaria* mats (cf. Thomsen *et al.*, 2009). Thus, it will be important to investigate the ecological role that *G. vermiculophylla* may play in enhancing the local faunal biodiversity and abundance, as has been done with other introduced macroalgae (Bulleri *et al.*, 2006; Olabarria *et al.*, 2009).

The capacity for vegetative propagation is fundamental to the success of some NIS (Nyberg & Wallentinus, 2005; Wright & Davis, 2006), leading to higher rates of successful colonization (Norton & Mathieson, 1983). *Gracilaria vermiculophylla* was always abundant and reproductive even though mature male gametophytes were never observed during the sampling period. This phenomenon has been reported for other red macroalgae (Yarish & Edwards, 1982).

There are different trends reported for the dominance of either the haploid or the diploid phases of *Gracilaria* in natural populations. A revision by Fierst *et al.* (2005) pointed to a dominance of the tetrasporophytes in the Gracilariales. For *G. vermiculophylla*, Rueness (2005) described a higher abundance of vegetative individuals in European waters. The different latitudinal, biological and physical processes might explain these differences (Thorner & Gaines, 2004). The patches we investigated in the Ria de Aveiro exhibited high variation in reproductive status, with some being almost always vegetative and others having a predominance of carposporophytic individuals. According to Norton & Mathieson (1983), vegetative propagation is a typical attribute of unattached seaweed populations. The fact that the patches with higher percentages of vegetative individuals were also the least stable seems to be in agreement with this view. The observed reproductive variability between patches might also be related to the different

abundances of gastropods (e.g. *Hydrobia ulvae*, *Littorina littorea*), since selective grazing of different maturation stages or reproductive structures has been reported for other red algae species (Thornber *et al.*, 2006; Vergés *et al.*, 2008).

The deviations from the typical *Polysiphonia*-type cycle (mixed cystocarpic and tetrasporic ramets) that we observed are similar to many others found in literature for *Gracilaria* (see reviews by Kain & Destombe, 1995; Polifrone *et al.*, 2006) and other red algae (Vera *et al.*, 2008). *In situ* germination of tetraspores is the probable explanation for gametophyte thalli being epiphytic on tetrasporophytes (Kain & Destombe, 1995).

Influence of environmental parameters on the development of G. vermiculophylla

Coastal lagoons/mudflats, such as the Ria de Aveiro, are stressful environments easily colonized by non-indigenous species (Occhipinti-Ambrogi & Savini, 2003). Extreme salinity and temperature fluctuations are common in these habitats and are favourable to species that can withstand wide variations of these factors (Yarish & Edwards, 1982; Yarish 1979; Lobban & Harrison, 1997). Despite the morphological similarities between the haploid and diploid phases, they may have different ecophysiological responses (e.g. release and germination of spores) to particular environmental factors (Luxoro & Santelices, 1989; Garza-Sánchez *et al.*, 2000). The results from the laboratory life-history studies agree with our field observations of reproductive material throughout the year and show that both the tetrasporophyte and carposporophyte phases of *Gracilaria vermiculophylla* are able to produce spores and germinate under a wide range of conditions. The highest germination occurred at 20°C under long-day conditions (16:8 h, L:D) for both phases. In the field, this corresponds to average conditions from spring to late summer. Tetraspores reached 100% germination rates in all conditions tested, but the same was not observed for carpospores. Differences in the success of carpospores vs tetraspores have already been reported for *G. pacifica* (Garza-Sánchez *et al.*, 2000).

The optimal growth performance of *G. vermiculophylla* was observed at 20°C for both the haploid and the diploid phases. These results are similar to other reports for this species (Yokoya *et al.*, 1999; Rueness 2005). On the other hand, Raikar *et al.* (2001) found that growth rates were higher at 25°C, with a tolerance limit of 35°C (RGR ~22% day⁻¹). However, our study confirms that several other factors must be taken into account in order to understand the

distribution and growth strategies of *G. vermiculophylla*. Photoperiod plays an important role, with longer days promoting higher growth rates and maturation of the plants. On the other hand, we found that under optimal temperature and photoperiod conditions, 40 μmol photons m⁻² s⁻¹ is enough to maximize the growth of *G. vermiculophylla*.

Differences in growth performances between the gametophyte and tetrasporophyte phases have been described for this (Yokoya *et al.*, 1999) and other *Gracilaria* species (Hoyle, 1978; Guimarães *et al.*, 1999). A reduction in growth rate is expected when thalli become reproductive (Santelices & Varela, 1995). From our growth experiments with the young sporelings, we observed higher RGR in the gametophytes than the tetrasporophytes (see Fig. 6). Nonetheless, in the experiment with older *Gracilaria* individuals, we did not find differences in the growth of the gametophyte and tetrasporophyte phases. The development of *G. vermiculophylla* spores was impaired at the lowest temperature tested (5°C), but it still took place, which can explain the presence of the species in northern Europe (Thomsen *et al.*, 2007). Like many other species (Norton & Mathieson, 1983), *G. vermiculophylla* may be able to 'perennate' as thallus fragments. However, spore recruitment of *G. vermiculophylla* at northern latitudes is limited to warmer months and this may have an effect in regulating the species' northern distribution. In its native Pacific region, *G. vermiculophylla* has an extensive latitudinal range (www.algaebase.org; Kim *et al.*, 2010) and multiple introductions of NIS have been reported, for example for *Asparagopsis armata* (Guiry & Dawes, 1992; Chualáin *et al.*, 2004). The low survival success of Ria de Aveiro *G. vermiculophylla* spores at 5°C could therefore reflect a different introduction from the western Pacific region than the populations in northern Europe: Kim *et al.* (2010) found that the same haplotype was common in populations of *G. vermiculophylla* in northern Europe and the probable original donor population but unfortunately, no samples were included from any southern European site; Rueness (2005) suggested that *G. vermiculophylla* reached northern Europe through secondary introduction from the south.

Final considerations

The introduction of a new species is generally an irreversible phenomenon, and when it develops a self-sustaining population, it is considered to be fully established in its recipient area (Boudouresque *et al.*, 2005). *Gracilaria vermiculophylla* is fully adapted to its non-native environment in the Ria de Aveiro estuary. Local

fisherman and former collectors of ‘moliço’ distinguish it perfectly from *Gracilariopsis longissima* and have already given each species a common name, leading us to believe that *Gracilaria vermiculophylla* may have been thriving in the area for more than four decades. Confirmation of this awaits molecular identification of old herbarium specimens, especially those currently labelled as *G. verrucosa*.

Gracilaria vermiculophylla is considered a habitat modifier (Wallentinus & Nyberg, 2007) and may increase the abundances of local fauna and flora (Nyberg *et al.*, 2009). On the other hand, it may have negative impacts on the local populations of the keystone seagrass species, *Zostera noltii*. Further ecological studies on the recipient community are warranted to understand the impact of *G. vermiculophylla* on *Z. noltii* and evaluate its invasiveness characteristics (Boudouresque, 2008). With a better understanding of the autoecology of *G. vermiculophylla*, it may be possible to develop management strategies to minimize any negative impacts of this NIS on native species (Freshwater *et al.*, 2006; Thomsen *et al.*, 2009). Encouraging the harvest of *G. vermiculophylla* as raw material for agar may help reduce its biomass (Villanueva *et al.*, 2009).

Gracilaria vermiculophylla is reproductive year round and attains high rates of germination and growth success under a wide range of environmental conditions. These facts help to explain its current high abundance in the Ria de Aveiro lagoon and are probably responsible for the widespread distribution of this cosmopolitan species.

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