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RESEARCH

Performance of the estuarine alga *Punctaria latifolia* (Phaeophyceae) under different abiotic culture conditions

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Abstract

In farming projects, environmental variations can affect growth, productivity, survival, and fertility, as well as the quality of the produced biomass. This is why estuarine algae could be a good alternative for developing aquaculture ventures in a global climate change scenario since they can develop in highly fluctuating environments with large temperature and salinity fluctuations and low light penetrability. In this study, the best culture conditions (*i.e.*, temperature; nutrients with Provasoli culture medium (PES) and PES + Kelpak®, a commercial biostimulant based on algae; salinity and photon flux) for the growth and fertility of gametophytes and sporophytes of the estuarine alga *Punctaria latifolia* were evaluated. Gametophytes and sporophytes of *P. latifolia* showed good tolerance to a wide range of abiotic variables. Under culture conditions, both stages presented the highest growth at intermediate to high temperatures (16—20 °C) and intermediate to high salinity (27.5 – 35 psu). However, gametophytes grew better at intermediate to high nutrient concentrations of PES and PES + Kelpak® and low and high light intensity (5—35 µmol photons m^{-2} s^{-1}), whereas sporophytes grew better at intermediate to low nutrients of PES and PES + Kelpak® and low light intensity (5 µmol photons m^{-2} s^{-1}). *Punctaria latifolia* would be a potentially cultivable alternative due to its broad tolerance to unfavorable environmental conditions in the climatic change we face.

Keywords Macroalgae · Cultivation · Growth · Fertility · Salinity levels · Biostimulant · Photon flux

Introduction

The seaweed industry has been growing at an accelerated rate due to its various applications in the food, cosmetics, pharmaceutical, organic fertilizer and biofuel industries, reaching a market size of 9.9 billion US$ in 2021. However, 75% of the global algal biomass is only produced by a small number of cultivable species, such as the brown algae *Laminaria japonica*, *Undaria pinnatifida* and *Sargassum fusiforme* and the red algae *Porphyra* spp., *Eucheuma* spp., *Kappaphycus alvarezii* and *Gracilaria* spp. (Cai et al. 2021; Naylor et al. 2021; Grand View Research 2022; FAO 2022). In this context, the expansion of macroalgae aquaculture requires the evaluation of new cultivable species with biotechnological potential, allowing the diversification and improvement of the sector and ensuring the sustainable management of natural populations in a changing environment.

Sea and/or hatchery culture projects require a comprehensive understanding of how fluctuations in environmental conditions influence the physiological responses of macroalgae (*i.e.,* growth rates, fertility or fecundity), as well as finding the appropriate environments for the settlement of cultivated populations (Wood et al. 2017; Campbell et al. 2019; Hurd et al. 2023). Added to this, the current context of climate change is causing an increase in ocean temperatures, an increase in nutrients loads in the water as a consequence of increased anthropogenic activity, and a decrease in salinity due to the increase in freshwater runoff (Fonselius and Valderrama 2003; Takolander et al. 2017; Ji and Gao 2021).

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For this reason, the industrial potential of the species, as well as its tolerance to different scenarios of cultivable environments, should be evaluated when considering cultivation enterprises.

In this situation, estuarine algae are proposed as an alternative for aquaculture ventures because these species have the ability to grow and reproduce in highly variable environments, where they are not only exposed to pulses of freshwater and seawater, but also to large temperature fluctuations, sedimentation pulses and lower light availability for photosynthesis (Kennish 2001; Borburema et al; 2021; Croce et al. 2021).

Among the brown estuarine algae, the genus *Punctaria* provides a compelling example of seaweeds with potential industrial uses, since extracts with antitumor and antioxidant activity have been obtained from members of this genus (Xu et al. 2004). Some research has identified unique sulfated polysaccharides present in *Punctaria* called xylofucan (Bilan et al. 2014a, b), which presented anticoagulant and antithrombotic effects similar to those of the heparinoid Clexane ® (enoxaparin) (Ustyuzhanina et al. 2016). Likewise, Poza et al. (unpublished) recently evaluated the alginate of *Punctaria* and demonstrated a significant antitumor effect on MCF-7 tumor cells.

Punctaria latifolia Greville is an intertidal brown alga widely distributed in temperate and cold coasts worldwide. This species was reported for the Arctic, Atlantic Islands, European coasts, North America, Asia, Australia and New Zealand (Guiry and Guiry 2022). In South America, *P. latifolia* has been reported on the coasts of Argentina and Uruguay (Guiry and Guiry 2022). *Punctaria latifolia* has a heteromorphic life cycle with alternating generations between a filamentous microthallus (gametophyte) and a macrothallus of flattened erect blades (sporophyte) (Gauna and Parodi 2010). The sporophytes, which can reach up to 15 cm in length, are simple, membranous ribbon-shaped with ruffled margins that arise from a small disc and grow singly or in groups (Asensi and Küpper 2012).

Previous studies have shown that this species can live in different environments, even being recorded in saline lagoons and estuaries with soft bottoms, where populations are tolerant to wide fluctuations in salinity, temperature, turbidity, and current speed (Kennish 2001; Thomsen and McGlathery 2006). Considering the ability of *P. latifolia* to grow and tolerate intrinsically variable habitats, this study aimed to evaluate the influence of different culture conditions that simulate a changing environmental scenario *(i.e.*, temperature, nutrient availability, salinity, and photon flux) on the settlement of spores and zygotes and the development of gametophytes and sporophytes.

To establish macroalgal cultivation, it is crucial to adjust the abiotic conditions to ensure the recruitment of spores and zygotes and guarantee the successful establishment of the early post-settlement phases. Although it is known how different conditions of temperature, radiation and concentration of artificial nutrients (such as Provasoli) affect the development of macroalgae in culture (Vadas et al. 1992; Bogaert et al. 2016; Camus and Buschmann 2017; Lind and Konar 2017; Poza et al. 2018, 2022; Suebsanguan et al. 2021); the effect of seaweed-derived biostimulants is not well known. However, recent studies have shown that algal biostimulants have growth-enhancing properties and are currently being tested as an alternative to complement or partially replace ingredients present in traditionally used culture media (Robertson-Andersson et al. 2006; Hurtado et al. 2012; Vatsos and Rebours 2015; Umanzor et al. 2020; Poza et al. 2022). In this context, an additional aim of the present study was to evaluate and compare the use of different concentrations of the artificial nutrients media Provasoli and its complementation with the algal bioestimulant Kelpak® on the settlement of spores and zygotes and the development of the post-settlement phases. Therefore, this study would lay the foundations for future cultivation endeavors of *P. latifolia*, where large-scale cultivation would be feasible in the variable environmental scenario that coastal environments face today on the southwest Atlantic coast.

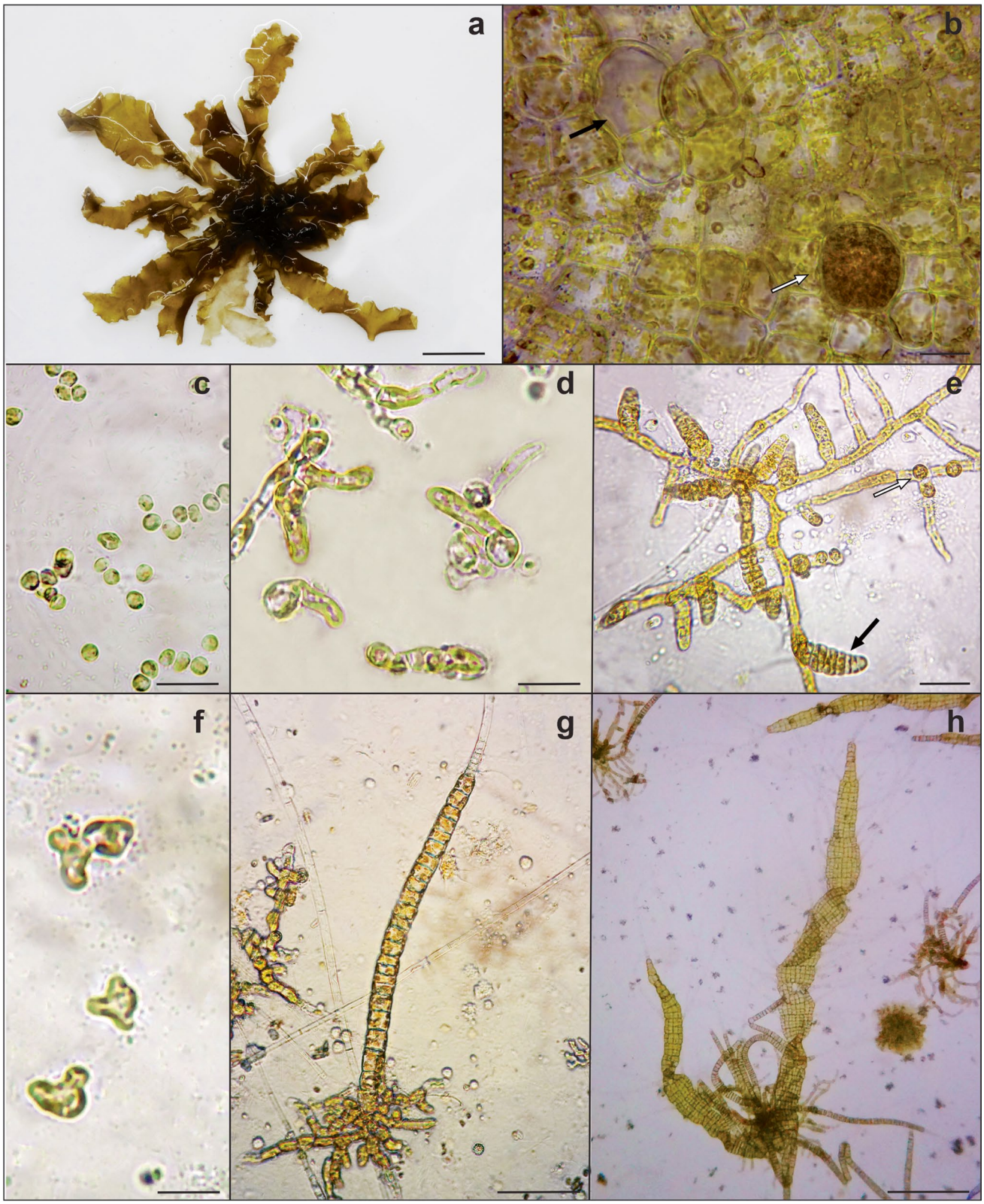
Materials and methods

Sample collection

Macroscopic sporophytes of *Punctaria latifolia* (Fig. 1a) were randomly collected from the salt marshes located in the Bahía Blanca Estuary, Argentina (38° 51′17″ S, 62°07′05″ W) during the coldest season, from June to September 2022, when the sporophytes developed in the nature (Fig. S1a-c). Once in the laboratory, the thallus size was measured (40 individuals) and mature thalli were selected to perform culture assays under different abiotic conditions.

The intertidal marsh in this area covers approximately 6 km^2 and extends for more than 1 km along the tidal gradient, with average water temperatures varying from 8 °C in winter to 22 °C in summer. The Bahía Blanca Estuary has been recognized as a nutrient-enriched environment, which usually maintains high levels of these inorganic compounds throughout the year (Freije and Marcovecchio 2004). Freshwater inputs into the estuary primarily originate from the Sauce Chico river and the Napostá stream. In addition, other intermittent contributions come from precipitations (350 mm to 1080 mm), with an average value of 550 mm (Celleri et al. 2018). Water salinity ranges between 15.84 in winter and 40.91 in summer, with an annual average of 33.98 (Angeletti and Cervellini 2015).

In this area, there is a constant exchange of suspended sediment along the entire front of the marsh, characterized



by a mixture of mud and sand (87% and 13%, respectively) with modal diameters of 12 and 130 µm, respectively (Pratolongo et al. 2010), and high levels of particulate organic matter (between 300 and 1000 mg Cm^{-3}), which generates high turbidity in the water column (Angeletti and Cervellini 2015).

◂**Fig. 1** Life cycle stages of *Punctaria latifolia* in culture. a. Macroscopic sporophytes collected from the natural habitat. Scale bar: 2 cm. b. Cross-section showing unilocular sporangia containing unispores (white arrow) and unilocular sporangia after spore release (black arrow) from sporophyte. Scale bar: 20 µm. c. Settled spores. Scale bar: 20 µm. d. Young microthalli with unipolar germination. Scale bar: 20 µm. e. Mature prostrate branched microscopic gametophyte with plurilocular gametangia (black arrow) and incipient gametangia (white arrow). Scale bar: 20 µm. f. Macrothalli germlings from zygotes showing lobed germination. Scale bar: 10 µm. g. Incipient formation of a macroscopic sporophyte. Scale bar: 20 µm. h. Young sporophyte. Scale bar: 200 µm

The sediment surface presents a striking morphology produced by elevated patches of bare mud between the vegetation and sediment deposits at higher elevations immediately seaward of the edge of the salt marsh. *Spartina alterniflora* Loisel is the dominant species in the lower section of the marsh that occurs as discontinuous patches (Pratolongo et al. 2009). Macroscopic sporophytic thalli of *P. latifolia* grow attached to the base of the *S. alterniflora* stems by means of a small disk, forming a belt surrounding the plant. The fronds of *P. latifolia* are 4 to 10 cm long and 1–2 cm wide (Fig. S1b-c).

Culture assays of *Punctaria latifolia* under different abiotic conditions

The sporophytes of *P. latifolia* collected in the field were transported at low temperatures, on damp paper towels and in black bags to avoid light. They remained at 6 ºC all night until starting the cultures the next day. Acclimatizing the fertile sporophytes in seawater is not advisable, because this induces premature sporulation (Oliveira et al. 1995; Redmond et al. 2014; Forbord et al. 2018). To start the cultures, the sporophytes were first cleaned with a brush and then immersed in freshwater with 0.5% chlorine for 10 s and rinsed with filtered and sterilized seawater. Treatment with surface disinfectants eliminates epiphytes, animals and microorganisms. Fertile sections of the sporophytes (0.5 mm^2) containing unilocular sporangia scattered on the thallus, were obtained under the microscope to initiate cultures (Fig. 1b).

Independent experiments were carried out with four different abiotic variables (*i.e.*, temperature, nutrients, salinity, and photon flux) whose levels were selected considering the range of variation of these variables in the area where the specimens of *P. latifolia* were collected. The different treatment conditions (Table S1) were maintained from the beginning of the culture with the release of the spores until the end of the experiment. The levels assigned to each treatment are as follows:

Temperature 10, 16, and 20 °C, with 10 mL L^{-1} of Provasoli culture medium (PES, Provasoli 1968), 35 psu, 5 µmol photons m^{-2} s^{-1}.

Nutrient conditions: three concentrations of the Provasoli culture medium (PES) of 5, 10, and 20 mL PES L^{-1}, hereafter referred to as P1, P2, P3; and three concentrations of PES + Kelpak® (Kelp Products Pty Ltd., Simon's Town, South Africa), for which 5 mL PES L^{-1} were supplemented with three different concentrations of Kelpak®: 1, 2, and 4 mL L^{-1}, following Robertson-Andersson et al. (2006), hereafter referred to as K1, K2, K3, at 16 ºC, 35 psu, and 5 µmol photons m^{-2} s^{-1}.
Salinity levels: 5, 12.5, 20, 27.5, and 35 psu, at 16 ºC, 10mL PES L^{-1}, and 5 µmol photons m^{-2} s^{-1}.
Photon flux: 5 and 35 µmol photons m^{-2} s^{-1}, at 16 ºC, 10mL PES L^{-1}, 35 psu.
The culture experiments were performed in 250 mL glass jars containing 0.5 mm^2-fertile fragments of sporophytes on a coverslip of 1.5 cm in diameter. The assays were performed in triplicate and evaluated during the 31-day incubation period. For all treatments, the light (L)/dark (D) regime was 12:12 h provided by cool white LED fluorescent tubes with an adjustable system to regulate the light intensities. The culture medium was refreshed every two days.

Spores and zygotes settlement were evaluated by counting the number of spores and fertilized gametes attached to the microscope slides between 12–24 h after release. The development of the thalli was also monitored, which made it possible to describe the life cycle under cultivation. The density of the gametophytes and sporophytes was measured by counting the viable individuals in an area of 3 cm^2, using a Nikon Eclipse TE 300 microscope (Japan) equipped with a Nikon FDX 35 camera. Also, the reproductive output of mature gametophytes was evaluated by counting the number of plurilocular gametangia of 10 gametophytes for each treatment. Furthermore, reproductive success was determined by establishing the relationship between the number of settled zygotes and the number of plurilocular gametangia per gametophyte. It is important to highlight that each gametophyte contains several plurilocular gametangia per thallus and each reproductive structure contains several locules that release more than one gamete into the environment. Finally, the size of 10 sporophytes generated in culture for each condition was measured using ImageJ v software. 1.46 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

To evaluate the number of spores and zygotes settled (number cm^{-2}), a generalized linear model with a Poisson error distribution (GLM) was used. To analyze the density of the gametophytes and sporophytes, generalized linear mixed models (GLMM) with Poisson error distribution were performed with the "lme4" package (Bates et al. 2015). To

analyze the reproductive output of gametangia according to the number of plurilocular gametangia per thallus, a Zero-inflated Negative Binomial (ZINB) model was conducted with the "lmtest" package (Zeileis and Hothorn 2002). The length reached by the sporophytes at the end of the treatment and the reproductive success were analyzed with a generalized linear model and (GLM) with a Gaussian error distribution using the "MASS" package (Venables and Ripley 2002). GLM and GLMMs were performed at a 0.05 significance level in R Studio. Using models (GLM, GLMM and ZINB) allowed for analyzing data that do not follow a normal distribution or are not independent without the need to apply transformations or non-parametric statistics, which are currently questioned.

Results

Life cycle of *P. latifolia*

Starting from the zoospores obtained from *P. latifolia,* the life cycle was completed in 31 days under laboratory conditions. The life cycle was heteromorphic, alternating between prostrate microscopic gametophytes (Fig. 1e) and erect macroscopic sporophytes (Fig. 1a and h). The fertile macrothallus of *P. latifolia* with unilocular sporangia (Fig. 1a-b) released biflagellate zoospores (5.7 ± 0.6 µm diameter) and they settled 12 to 24 h after their release (Fig. 1c). Once settled, they exhibited unipolar germination and developed a germ tube. Later, transversal divisions formed branched uniseriate filaments, which developed into a prostrate filamentous microscopic gametophyte bearing hyaline hairs (Fig. 1d, e). After two or three weeks of incubation, terminal and intercalary sessile plurilocular gametangia formed on the branches of the microscopic gametophytes (Fig. 1e). The gametophytes formed flagellated gametes that produced a zygote. The zygote settled and germinated with two or more lobes, forming a branched protonema (Fig. 1f). Then, one to several young laminar thalli emerged from the diploid protonema (Fig. 1g-h).

Culture assay of *P. latifolia* under different abiotic conditions

The culture experiments revealed differences in the number of settled spores and zygotes, as well as the subsequent development of gametophytes from spores, the reproductive output of the gametophyte, the reproductive success, and the development of sporophytes from zygotes, according to temperature, nutrient concentration, salinity level and photon fluxes (Table 1). Under either condition, the total average density of settled spores (128 ± 7 individuals cm^{-2}) was significantly higher than the density of settled zygotes (13 ± 3 individuals cm^{-2}) (*df: 1,* χ^2*: 106,* $p < 0.0001$). Likewise, the average gametophyte density (12 ± 20 individuals cm^{-2}) was significantly higher than the sporophyte density (7 ± 12 individuals cm^{-2}) (*df:* 1, χ^2: 519.93, $p < 0.0001$). The density of gametophytes was higher at the beginning of the experiment, with a progressive decrease as the incubation time progressed (Fig. 2–5, Table 1). This decrease in the number of gametophytic thalli was related to the mortality of early life stages and, subsequently, to the formation of sporophytes developed from the fertile gametophytes during the last two weeks of incubation.

Temperature experiment The temperature had a significant effect on spore and zygote settlement ($p < 0.0001$) (Table 1). The highest density of settled spores was recorded at 16 ºC (99 ± 10 spores cm^{-2}), and zygotes at 20 ºC (54 ± 10 zygotes cm^{-2}), while the lowest values were recorded at 10 ºC for both spores (44 ± 19 spores cm^{-2}) and zygotes (3 ± 2 zygotes cm^{-2}) (Fig. 2a). In the same way, the temperature of the culture throughout incubation time had a significant ($p < 0.0001$) effect on gametophyte density, gametophyte reproductive output and sporophyte density (Table 1). A progressive decrease in gametophytes was observed throughout the entire experiment, although the decrease was less significant with increasing temperature, with the highest final density at 20 °C (Fig. 2b). The reproductive output of gametophytes was also higher at 16 and 20 ºC than at 10 ºC, reaching values of 27 ± 3 and 28 ± 3 plurilocular gametangia per gametophyte, respectively, during the third week of incubation (between 16–23 days), and showed a decrease at the end of the incubation period (Fig. 2c). However, reproductive success did not present significant differences between the different temperatures evaluated, with an average of 1.74 ± 0.74 zygote per gametangia (*df: 2, F: 0.14,* $p = 0.868$). In the high-temperature treatment (20 °C), the gametophytes managed to survive, producing a significantly higher sporophyte density with 51 ± 10 thalli cm^{-2} (Fig. 2b). However, the vegetative growth of the sporophytes did not vary significantly between the three temperatures tested, showing tolerance to high temperatures (Table 1), reaching between 1.7–2 mm at the end of the incubation period after 31 days (Fig. 2d).

Nutrient conditions The nutrient concentrations used for the cultures had a significant ($p < 0.0001$) effect on the settled spores and zygotes (Table 1). The highest density of settled spores was recorded at the P2 treatment (101 ± 6 spores cm^{-2}; Fig. 3a) and K3 concentrations (111 ± 30 spores cm^{-2} Fig. 3b). In contrast, the zygote density did not show significant differences between the three PES treatments (Fig. 3a). However, in the case of supplementation with Kelpak® the zygote density was significantly higher at K1 concentration (40 ± 18 zygotes cm^{-2}), being harmful at higher concentrations (Fig. 3b).

Table 1 Summary statistics GLM analysis testing the temperature (T), nutrient concentration (PES) and (PES + KELPAK), salinity (S) and photon irradiance (Pf) on the number of spores and zygotes settled and on sporophyte size after 31 incubation days of *P. latifolia*. GLMM analysis testing the effects of incubation times (t) and the different variables (T, PES, PES + KELPAK, S, Pt) on the number of sporophytes and gametophytes. ZINB analysis testing the different variables (t, T, PES, PES + KELPAK, S, Pt) on the number of plurilocular gametangia per gametophyte. *df,* degrees of freedom, *p values* (< 0.05), probability

	T	(PES)	(PES + KELPAK)	S	Pf	t	T × t	(PES) × t	(PES + KELPAK) × t	S × t	Pf × t
GLM. N° spores											
df	2	2	3	4	1						
Statistic (χ^2)	763.9	840.5	328.5	311.9	357.6						
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001						
GLM. N° zygotes											
df	2	2	3	4	1						
Statistic (χ^2)	147.96	3.45	437.64	1113.95	1448.88						
p-value	< 0.0001	0.1257	< 0.0001	< 0.0001	< 0.0001						
GLM. Sporophyte size after 31 incubation days											
Length of blade per thallus											
df	2	2	3	4	1						
Statistic (χ^2)	0.24	0.29	9.86	10.01	16.03						
p-value	0.7876	0.7887	0.0022	0.00013	< 0.0001						
GLMM. Gametophytes density											
df	2	2	3	4	1	3	6	6	9	12	3
Statistic (χ^2)	335.6	17.45	25.84	1968.8	123.4	10,916.4	151.3	32.7	20.55	388.5	76.2
p-value	< 0.0001	0.0077	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0017	< 0.0001	< 0.0001
GLMM. Sporophytes density											
df	2	2	3	4	1	1	2	2	3	4	1
Statistic (χ^2)	750.1	24.16	36.07	830.4	1016.71	54.42	92.43	39.43	9.8	73.53	1.17
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0202	< 0.0001	0.2795
ZINB. Reproductive output											
N° gametangia per thallus											
df	2	2	3	4	1	3	6	6	9	12	3
Statistic (χ^2)	367.45	149.01	162.83	327.9	332.81	124.88	233.97	710.03	187.33	189.13	205.03
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0017	< 0.0001	< 0.0001	< 0.0001	< 0.0001

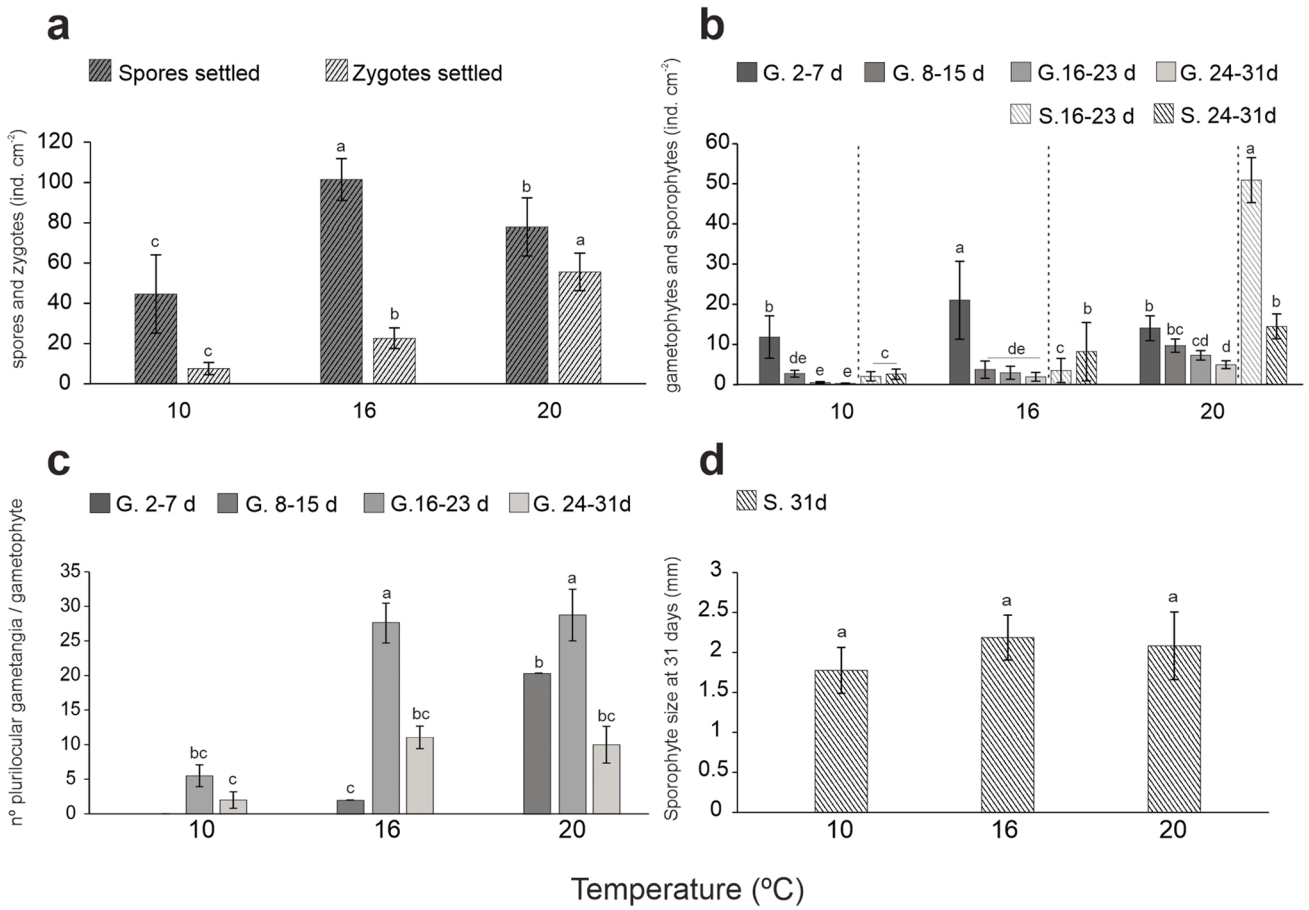
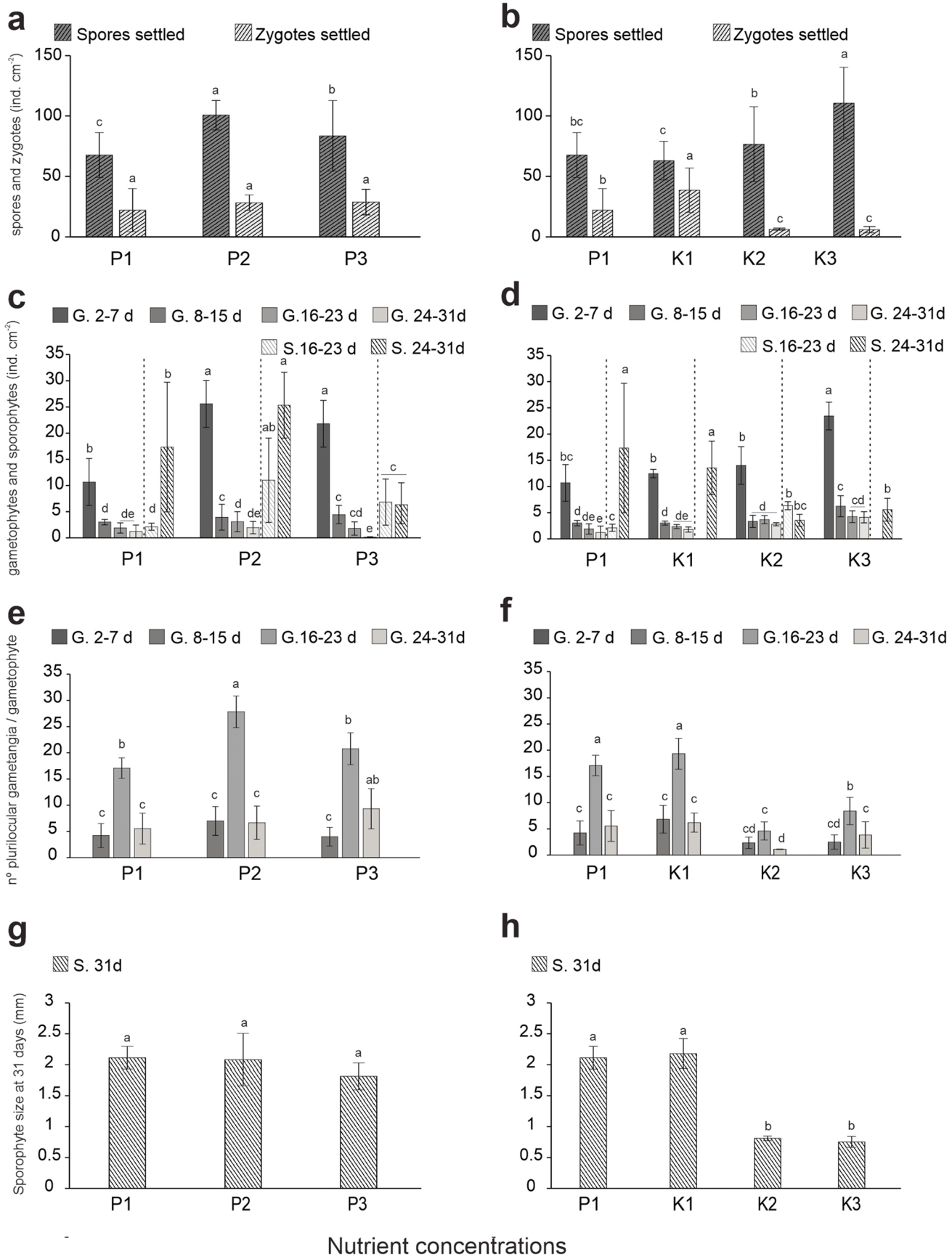


Fig. 2 Data obtained from *P. latifolia* incubated under 10, 16 and 20 °C, for 31 days. (a) Settlement of spores and zygotes. (b) Density of gametophytes and sporophytes at different incubation times. (c) Reproductive output of gametophytes. (d) Size achieved by the sporophytes at the end of the experiment. Vertical lines represent mean ± standard deviation, n = 3 (number of independent replicates). Significant differences ($\alpha < 0.05$) are indicated by different letters using Tukey's Honestly Significant Difference (HSD). Symbols: G (gametophytic individuals), S (sporophytic individuals), d (days)

Nutrient concentrations also had a significant effect ($p < 0.0001$) on gametophyte density, gametophyte reproductive output and sporophyte density (Table 1). The highest densities of gametophytes were recorded during the first week of incubation at the P2, P3, (21 ± 10; 22 ± 4 individuals cm^{-2}, respectively, Fig. 3c) and K3 concentration (24 ± 2 individuals cm^{-2}, Fig. 3d). The reproductive output was higher at the P2 concentration with 28 ± 3 plurilocular gametangia per gametophyte, during the third week of incubation (between 16–21 days, Fig. 3e), while for the treatments with Kelpak® supplementation it was higher at K1 concentration, with 19 ± 6 plurilocular gametangia per gametophyte during the third week of incubation (between 16 and 21 days, Fig. 3f), however, no significant differences were observed with the control corresponding to P1. Reproductive success was similar for the different PES concentrations, with an average of 1.98 ± 0.48 zygotes per gametangia (*df: 2, F: 1.58,* $p = 0.177$), and for different levels of Kelpak® supplementation and P1, with an average of 1.25 ± 0.85 zygotes per gametangia (*df: 3, F: 0.136,* $p = 0.982$).

The highest densities of sporophytes were attained at intermediate nutrient concentrations, at P2 concentration (25 ± 5 individuals cm^{-2}, respectively) and, in the case of supplementation with Kelpak®, at the lowest concentration K1 (14 ± 5 individuals cm^{-2}), being harmful at higher concentrations of Kelpak®. The concentration of nutrients had a differential effect on the vegetative growth of sporophytes depending on the treatment used (PES or PES + Kelpak®) (Table 1). No significant differences were observed between the different concentrations of PES used. However, in the case of supplementation with Kelpak®, the largest sporophytes were recorded at the lowest concentration of supplementation K1 and in the control P1, reaching sizes between 2—2.2 mm (Fig. 3g-h, Table 1).

Salinity levels Spores and zygotes settled over a wide range of salinities from 35 to 20 psu, but showed significantly


a
Spores settled
Zygotes settled
spores and zygotes (ind. cm-2)
P1
P2
P3
b
Spores settled
Zygotes settled
P1
K1
K2
K3
c
G. 2-7 d
G. 8-15 d
G.16-23 d
G. 24-31d
S.16-23 d
S. 24-31d
gametophytes and sporophytes (ind. cm-2)
P1
P2
P3
d
G. 2-7 d
G. 8-15 d
G.16-23 d
G. 24-31d
S.16-23 d
S. 24-31d
P1
K1
K2
K3
e
G. 2-7 d
G. 8-15 d
G.16-23 d
G. 24-31d
nº plurilocular gametangia / gametophyte
P1
P2
P3
f
G. 2-7 d
G. 8-15 d
G.16-23 d
G. 24-31d
P1
K1
K2
K3
g
S. 31d
Sporophyte size at 31 days (mm)
P1
P2
P3
h
S. 31d
P1
K1
K2
K3
Nutrient concentrations

◂**Fig. 3** Data obtained from *P. latifolia* incubated under three PES and PES + Kelpak concentrations, for 31 days. (a) Settlement of spores and zygotes. (b) Density of gametophytes and sporophytes at different incubation times. (c) Reproductive output of gametophytes. (d) Size achieved by the sporophytes at the end of the experiment. Vertical lines represent mean ± standard deviation, n = 3 (number of independent replicates). Significant differences ($\alpha < 0.05$) are indicated by different letters using Tukey's Honestly Significant Difference (HSD). Symbols: G (gametophytic individuals), S (sporophytic individuals), d (days)

lower values under lower salinities (Table 1). The highest settlement values recorded at 27.5 psu (169 ± 17 and 62 ± 75 individuals cm^{-2}, respectively) (Fig. 4a). The effect of salinity throughout the incubation time had a significant ($p < 0.0001$) effect on gametophyte density, gametophyte reproductive output and sporophyte density (Fig. 4b-d, Table 1). The gametophytes and sporophytes could grow in various salinities throughout the incubation period (Fig. 4b). The highest gametophyte densities were recorded during the first week of incubation in salinity concentrations of 27.5 to 20 psu (29 ± 4 individuals cm^{-2} and 31 ± 4 individuals cm^{-2}, respectively). During the third week of cultivation, the reproductive output of gametophytes was higher at 35 and 27.5 psu, reaching values of 23 and 24 plurilocular gametangia per gametophyte, respectively (Fig. 4c). Reproductive success was greater at 27.5 psu with 8.66 ± 5.78 zygotes per gametangia, followed by 35 and 20 psu with 4.33 ± 5.78 and 4.00 ± 2.08 zygotes per gametangia, respectively. The lowest reproductive success was at 12 psu with 1.18 ± 0.8 zygotes per gametangia, whereas at 5 no success was recorded (*df: 3, F: 3.864, p* = 0.013). A higher sporophyte density was recorded at 27.5 psu during the last week of culture (15 ± 12 individuals cm^{-2}) (Fig. 4b). Likewise, the largest sporophytes were recorded at 27.5 psu, reaching 3 mm (Fig. 4d). In general, under the lowest salinities evaluated (5 and 12 PSU), all measured variables showed a significantly reduced performance (Fig. 4a,b,c,d).

Photon flux The photon flux significantly affected ($p < 0.0001$) the settlement of spores and zygotes (Table 1, Fig. 5a). While spore settlement was favored in the higher light treatment (35 µmol photons m^{-2} s^{-1}), zygotes only settled and developed successfully in the low photon flux treatment (5 µmol photons m^{-2} s^{-1}; Fig. 5a). Similarly, radiation also had a significant effect ($p < 0.0001$) on the density of gametophytes and sporophytes over time (Fig. 5b). Initially, gametophytes were able to develop under both low and high photon fluxes (5–35 µmol photons m^{-2} s^{-1}), exhibiting higher numbers during the first week of incubation under low and high photon flux conditions (18 ± 8 and 24 ± 11 individuals cm^{-2}, respectively) (Fig. 5b). However, as time progressed, the sustained high light intensity became stressful, leading to decreased gametangia production and subsequently limiting sporophyte formation (Fig. 5c). on (Fig. 5c). Reproductive success occurred mainly at low light intensities with 2.56 ± 2.23 zygotes per gametangia, while at high light intensities, it was 0.28 ± 0.1 zygotes per gametangia (*df:* 1*, F: 4.69, p* = 0.036). Sporophytes were significantly more abundant under low photon flux conditions (11 ± 7 individuals cm^{-2}), reaching sizes of up to 2.3 ± 0.2 mm in height. In contrast, under the high photon flux treatment, sporophytes failed to develop erect thalli, rendering it impossible to measure their growth.

Discussion

Punctaria latifolia culture, initiated from spores originating from reproductive tissue with unilocular sporangia, developed a heteromorphic life cycle under laboratory conditions, with alternation of prostrate microscopic gametophytes and erect macroscopic sporophytes, which completed the life cycle in 31 days. Previous studies on this species have revealed that it also presents a direct cycle occurring at 21 °C, photon flux of 140 µmol photons m^{-2} s^{-1} and photoperiod of 12:12 light/dark (Clayton and Ducker 1970; Gauna and Parodi 2010), this direct cycle involves the release of spores by plurilocular sporangia, which then generate new macrothalli. However, in the present study, we did not find any plurilocular sporangia in the fertile sporophytes collected from the salt marsh from Bahía Blanca Estuary.

The absence of plurilocular sporangia on sporophytes of *P. latifolia* may be related to the time of the year when the specimens were collected (spring) to start the cultures. In the marshes of the Bahía Blanca Estuary, sporophytes of *P. latifolia* are present during winter and early spring, while during summer and autumn, they remain in its gametophytic phase. In heteromorphic life cycles, the sporophytes can contain uni- and plurilocular sporangia. The plurilocular sporangia generate diploid spores that guarantee the permanence of the macroscopic sporophyte by regenerating the same macroscopic stage during favorable conditions. In contrast, unilocular sporangia generate haploid spores by meiosis and guarantee the next generation represented by microscopic gametophytes. In this way, a greater load of unilocular sporangia in sporophytic thalli before the transition from a sporophytic to gametophytic generation could explain the absence of plurilocular sporangia in the thalli used to start the cultures as they were collected at the end of the sporophyte season. This fact has been demonstrated in another brown alga, *Leathesia marina*, where a greater abundance of unilocular sporangia was observed at the end of the macroscopic phase season (Poza et al. 2017). However, it is necessary to carry out phenological and population studies of *P. latifolia* to explore this hypothesis.

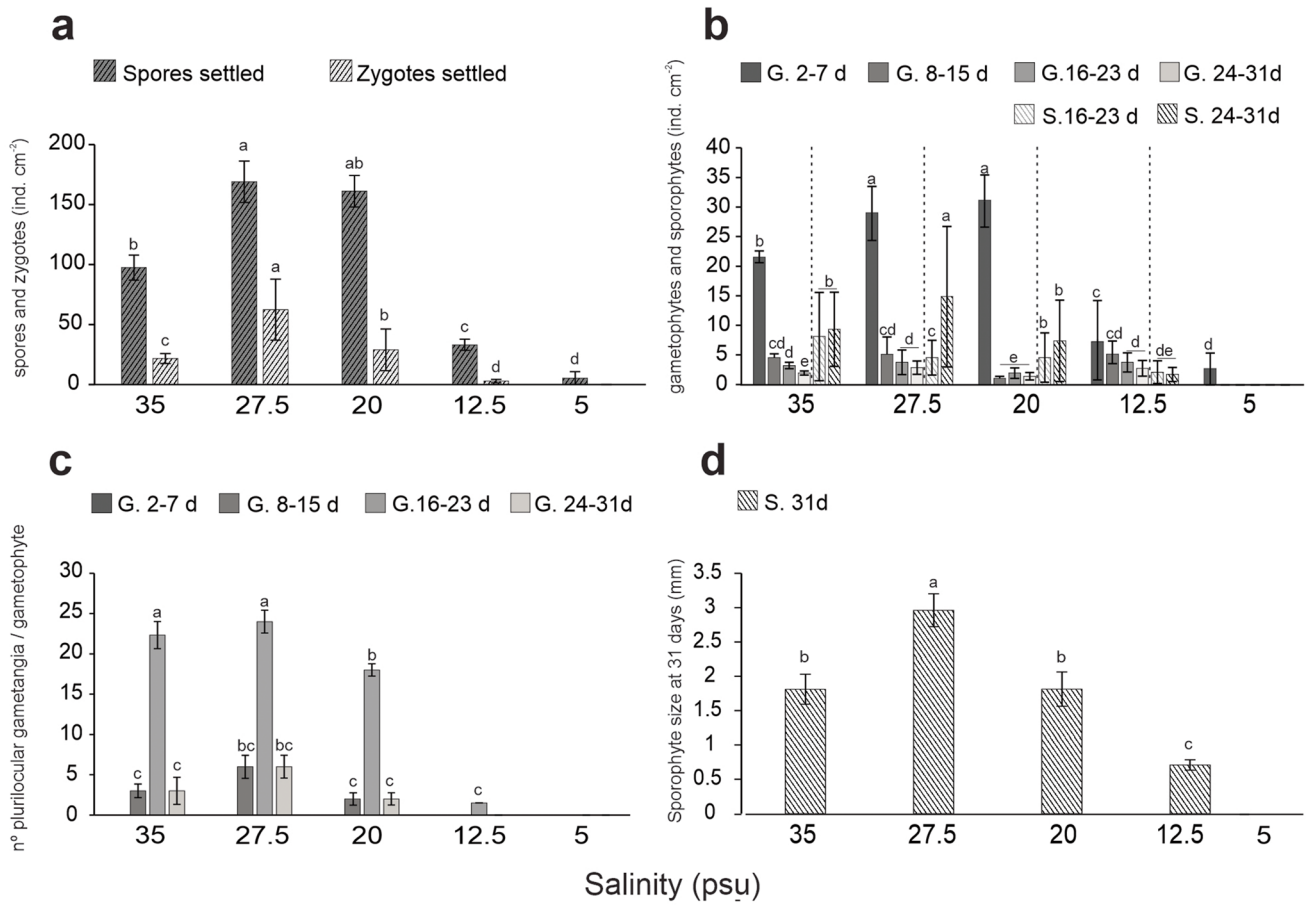


Fig. 4 Data obtained from *P. latifolia* incubated under 5, 12.5, 20, 27.5, 35 PSU salinity, for 31 days. (a) Settlement of spores and zygotes. (b) Density of gametophytes and sporophytes at different incubation times. (c) Reproductive output of gametophytes. (d) Size achieved by the sporophytes at the end of the experiment. Vertical lines represent mean ± standard deviation, n = 3 (number of independent replicates). Significant differences ($\alpha < 0.05$) are indicated by different letters using Tukey's Honestly Significant Difference (HSD). Symbols: G (gametophytic individuals), S (sporophytic individuals), d (days)

In cultivation ventures, early mortality of spores and zygotes after settlement could be a bottleneck in the successful recruitment of macroalgae used for cultivation purposes (Vadas et al. 1992; Poza et al. 2018, 2022). In this study, the density of settled spores was higher than that of settled zygotes due to non-fertilization or mortality, as has been previously described (*e.g.*, Lin et al. 2008; Poza et al. 2018, 2022). Also, early mortality of settled spores and gametophyte germlings occurred in all the treatments during the first week. Then, gametophyte density decreased slowly while the number of sporophytes began to increase. This pattern occurs in ephemeral algae that have heteromorphic life cycles, such as *P. latifolia*, *Leathesia marina*, *Colpomenia peregrina*, *Asperococcus ensiformis* (De Wreede and Klinger 1988; Poza et al. 2018, 2022), which show ecological differences between a stage resistant to biotic or abiotic stress (microthalli) and an ephemeral stage of rapid growth (macrothalli), which appears when seasonal conditions are suitable (Carney and Edwards 2006; Vergés et al. 2008). These ephemeral algae are characterized by opportunistic life strategies, with high growth rates and reproductive capabilities which allow them to respond rapidly to different environmental stress conditions (Agrawal 2012). Thus, the life cycle and reproductive patterns might be the outcome of adaptations to a seasonally changing environment (Roleda 2006; Véliz and Edding 2006; Gauna et al. 2013).

The evaluation of the life cycle of *P. latifolia* under different abiotic growth conditions (temperature, nutrients, salinity, and photon flux) allowed us to find differential patterns of settlement of spores and zygotes and the subsequent development and growth of gametophytes and sporophytes over time. Abiotic variables are known to affect spore and zygote settlement and thus directly contribute to the variability of macroalgae communities over time (Lind and Konar 2017). The temperature had a differential effect on the settlement of spores and zygotes and the development of gametophytes and sporophytes. Medium and high temperatures (16 and 20 ºC) favored the survival of spores and the subsequent development

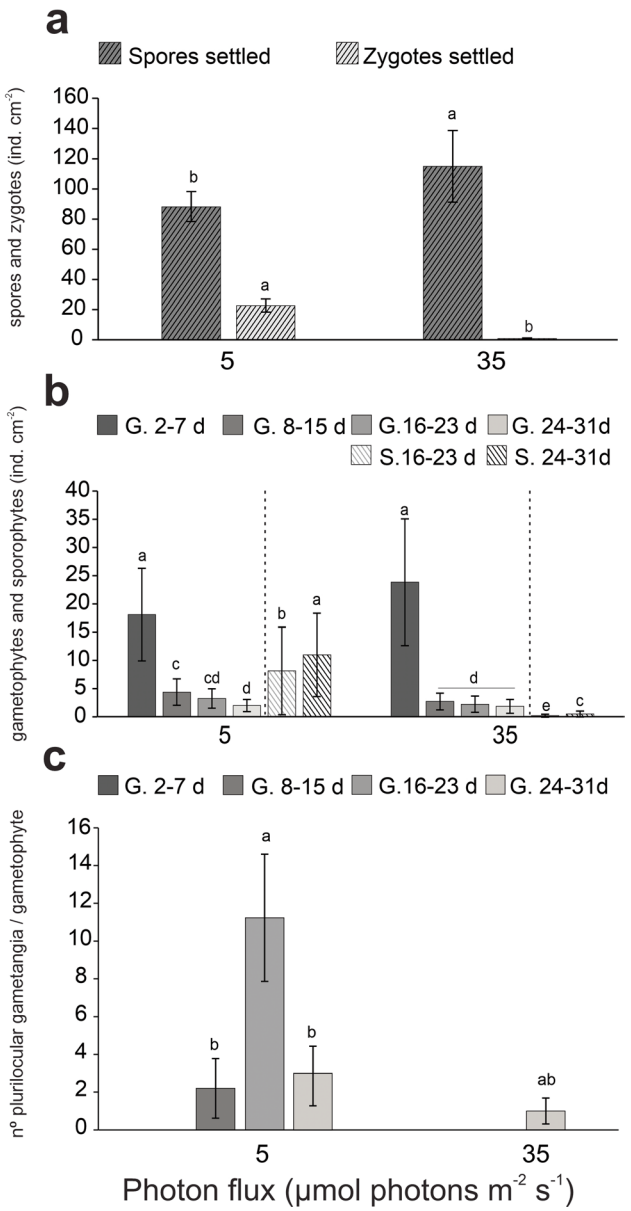


Fig. 5 Data obtained from *P. latifolia* incubated under 5 and 35 µmol photons m^{-2} s^{-1} of photon fluxes, for 31 days. (a) Settlement of spores and zygotes. (b) Density of gametophytes and sporophytes at different incubation times. (c) Reproductive output of gametophytes. Vertical lines represent mean ± standard deviation, n = 3 (number of independent replicates). Significant differences ($\alpha < 0.05$) are indicated by different letters using Tukey's Honestly Significant Difference (HSD). Symbols: G (gametophytic individuals), S (sporophytic individuals), d (days). (*) In the intense light treatment, sporophytes did not form an erect thallus, making it impossible to measure their growth

of gametophytes, reflected in greater density and reproductive success. This would explain the higher density of sporophytes at 20 ºC. These results indicate that although *P. latifolia* can grow and reproduce in a temperature range between 10–20 ºC, the highest densities were favored by intermediate and high temperatures. Under natural conditions, this species is widely distributed on the Argentine Atlantic coast (38º to 47º S), where temperatures range between 5 ºC and 21 ºC (Asensi and Küpper 2012). In the marshes, temperatures range between 8 ºC and 16 °C during winter and early spring when *P. latifolia* sporophytes are present. On the contrary, during the summer and autumn months, temperatures range between 16 and 22 °C, when the algae persist in their gametophytic phase (Capelli de Steffens and Campo de Ferreras 2004; Freije et al. 2008). Haplodiplontic life cycles allow species to occupy seasonally variable environments (Hughes and Otto 1999; Krueger-Hadfield 2020). Although this could be an evolutionary advantage in the face of climate change, if the haploid phase is exposed to unfavorable conditions, future sexual reproduction may not occur and the life cycle will remain interrupted and uncoupled. In this way, species that tolerate greater climatic amplitudes in both life cycle phases will be favored.

The nutrients used in culture also influenced the *P. latifolia* development. The density of settled spores was favored by intermediate concentrations of PES (P2) and high supplementation with Kelpak® (K3). On the contrary, greater supplementation with Kelpak® had a detrimental effect on the settlement and development of zygotes, making traditional culture medium (PES) more convenient.

Although the spores do not require nutrients in order to settle, several studies have shown that they can react to their chemical environment through chemostatic behavior. This adaptation could allow seaweed spores to find and settle in a suitable microhabitat for gametophytic growth (Amsler and Neushul 1989; Amsler and Fairhead 2005). Several studies have also shown that gametogenesis could be induced by specific nutrient requirements, where excesses might inhibit the process (Brawley and Johnson 1992; Boderskov et al. 2021). This could explain the detrimental effect of high Kelpak® supplementation on zygote settlement and sporophytes growth. In the same way, the development of gametophytes throughout the incubation time was favored by intermediate and high concentrations of PES and PES + Kelpak®. However, the highest sporophyte density was achieved at intermediate and low nutrient concentrations of PES and PES + Kelpak®. This differential adaptation to the nutritional requirements of each stage of the life cycle could be stimulated by the natural environment. Negrín et al. (2011) have shown that pulses of nitrogen vary throughout the year in the salt marshes where *P. latifolia* was collected, with an increase during spring and summer (Negrin et al. 2011). This would explain the greater tolerance of gametophytes to the high concentrations of nutrients present mainly during the summer, while sporophytes are found during the period of lower nutrient concentrations in the natural environment. Although it appears that both phases of the life cycle of *P. latifolia* presented differential preferences in nutrient

requirements, there is an overlap or dependence of the haploid or diploid stage on the other stage in terms of nutrients and, as such, they do not constitute distinct ecological entities. This could explain that although the gametophyte develops well at intermediate and high concentrations, the greatest reproductive efficiency was at intermediate concentrations coinciding with the sporophyte requirements under natural conditions.

On the other hand, the results of this study suggest that supplementation with Kelpak® would reduce the use of Provasoli medium for the development of gametophytes. In the same way, recent studies also tested the efficacy of supplementation with seaweed-based biostimulants, like Kelpak®, in cultures of *Eucheumatopsis isiformis*, *Asperococcus ensiformis*, *Ulva lactuca* and *Gracilaria gracilis,* obtaining favorable results (Robertson-Andersson 2006; Poza et al. 2022; Umanzor et al. 2020). The efficacy of Kelpak® has been attributed to its content of phytohormones, including auxins and cytokinins, as well as the content of alginate, amino acids, and small amounts of macro and microelements (Szczepanek and Siwik-Ziomek 2019). Thus, in addition to the positive effects of seaweed extracts on land plant-growth-enhancing properties, together with increased disease resistance and tolerance to climatic stresses, such as cold or drought (Khan et al. 2009; Briceño-Domínguez et al. 2014; Arioli et al. 2015), the application of seaweed extracts could also improve the growth, yields, and overall vigor of seaweed culturing (Souza et al. 2018; Hurtado and Critchley 2020).

The salinity experiment revealed that spores and zygotes settled in a wide range of salinities from 35 to 20 psu, recording the maximum settlement values at 27.5 psu. Similarly, the gametophytes and sporophytes were able to grow in a wide range of salinity throughout the incubation time. However, both gametophytes and sporophytes were less tolerant to low salinities of 12 and 5 psu, where the settlement of spores and zygotes and the subsequent development of thalli were notably reduced. Ecophysiological studies on the effects of salinity on algal growth revealed that intertidal seaweeds can tolerate wide salinity ranges, from low (5–10 psu) to twice the normal seawater salinity (66–68 psu) and, in most cases, with optimal growth under normal seawater conditions (33–37 psu) (Larsen and Sand-Jensen 2006; Karsten 2012; Diehl et al. 2020). Certain genera, including *Punctaria* and others such as *Ulva*, *Cladophora* and *Ceramium*, can also grow in estuarine regions and soft bottom marshes, which are considered stressful habitats due to the lack of hard substrates, low light penetration due to the excess of sediments, variation in salinity, pollution, competition and grazing factors, favoring the settlement of annual species (Larsen and Sand-Jensen 2006; Croce et al. 2021). In the natural environment, *P. latifolia* is exposed to freshwater pulses from the different tributaries that enter the Bahía Blanca Estuary, which would explain its wide tolerance to fluctuating salinity conditions.

Photon flux affected the settlement of spores and zygotes differently. While spore settlement was favored by a high photon flux of 35 µmol photons m^{-2} s^{-1}, zygotes only settled and developed at a low photon flux of 5 µmol photons m^{-2} s^{-1}. Likewise, although gametophytes developed at both high and low photon fluxes, sporophytes developed only at low light intensity. They grew better at the low photon flux since they did not develop an erect thallus at the high photon flux. This occurred because, although gametophytes could develop with high light intensities, this condition limited the formation of reproductive structures, preventing the formation of sporophytes. These results could be related not only to the seasonality, since under natural conditions the sporophytes appear in nature during the winter when the solar exposition is lower, but also as an adaptation to large amounts of sediment in the environment. Furthermore, although the association between *P. latifolia* and *S. alterniflora* may limit the load of suspended solids when the tide rises, the excess sediment covers the algae and, on occasions, they also tend to be partially buried by the sediment retained by the plants (Croce et al. 2021). Additionally, the gametophyte stage possibly exhibits greater resistance to high light and fluctuating salinity conditions, as demonstrated in the present study. This ability allows the species to survive during the summer months when exposed to higher temperatures and desiccation.

The responses of estuarine macroalgae, such as *P. latifolia*, to changes in environmental conditions are considered in the context of the high variability inherent to estuarine habitats. In addition to this, the species that live in the intertidal region are exposed to tidal and wave regimes, hydrodynamics and salinity balance, and geomorphological evolution (Ducrotoy et al. 2019). Therefore, it could be inferred that these conditions give the species inhabiting these ecosystems a greater degree of resistance and resilience to environmental change, being able to adapt to climate change.

Specifically, in the Bahía Blanca Estuary, as has already been mentioned, the macroscopic sporophyte thalli of *P. latifolia* have always been found associated with the salt marshes of *S. alterniflora*, forming a belt surrounding the plants. This association between *P. latifolia* and *S. alterniflora* could favor the development of *P. latifolia* by mitigating the adverse conditions. First of all, *S. alterniflora* provides a substrate for the development of *P. latifoilia*, because the plants grow from rhizomes in a radial structure and as these circles grow, they fuse generating a solid structure (Perillo 2019). In addition, stems and leaves attenuate currents and wave energy, which protects *P. latifolia* from erosion and provide protection against desiccation by increasing shading (Pratolongo et al. 2019). Studies carried out in the same area have

revealed that in the sites dominated by *S. alterniflora*, there was a lower concentration of total suspended solids than in the bare marsh (Pratolongo et al. 2010), allowing higher light availability and preventing *P. latifolia* from becoming buried. This occurs because the presence of plants reduces the turbulence generated by waves and currents, helping with the deposition of transported sediments (Leonard and Luther 1995; Pratolongo et al. 2019). Furthermore, nutrients are deposited along with the sediment, which are used by plants for their growth and by other species associated with them such as *P. latifolia*. For this reason, the marshes formed by *S. alterniflora* could improve estuary conditions in that region regarding water quality and sediment sequestration and, as a temperature regulator, mitigate environmental changes, which are commonly highly stressful in estuaries.

In conclusion, this study allowed us to lay the foundation for future cultivation efforts of *P. latifolia*, since it was possible to determine the conditions for promoting the growth of gametophytes and sporophytes under controlled conditions. The cultivation of *P. latifolia* is a promising new potentially cultivable resource for obtaining bioactive products of commercial interest (Xu et al. 2004; Ustyuzhanina et al. 2016). Additionally, due to its tolerance to a wide range of environmental conditions (temperatures of 10 to 20 ºC, high and low levels of nutrients, salinities of 20 to 35 psu and photon flux of 5 to 35 µmol photons m^{-2} s^{-1}), this species offers an opportunity for future aquaculture enterprises, enabling diversification within the macroalgae cultivation sector, which is currently limited to a few species, and it offers an alternative for the use of estuarine environments for the cultivation of tolerant species. However, there are still several challenges to the development of large-scale commercial cultivation of this species that require attention, so it is essential to evaluate the seed-rope method for the development of sporophyte and gametophyte thalli that can be taken out to sea.

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Authors' contributions Poza, Ailen M.: Conceptualization, Methodology, Sample processing, Formal analysis, Writing - Original Draft, Review and Editing, Visualization. Carolina Fernández: Review and Editing. M. Emilia Croce: Review and Editing. M. Cecilia Gauna: Resources, Review and Editing, Project administration, Alejandro H. Buschmann: Resources, Review and Editing, Supervision, Project administration.

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Data Availability All data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, for any investigator who requires it.

Declarations

Competing interests The authors declare no competing interests.

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