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Biochemical properties and culture optimization of *Leathesia marina* (Phaeophyceae)

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ABSTRACT

In recent years, interest in the chemical composition of algae, and the art of farming them, has grown due to their nutritional and health benefits. Species of *Leathesia* collected from nature have pharmaceutical properties, such as antitumor, antiviral, antioxidant and cytotoxic activity. Accordingly, their culture under controlled conditions is fundamental for the sustainable maintenance of natural populations. In this study, the biochemical constituents of *L. marina*: organic matter, alginate, total carbohydrate, protein content, pigments, polyphenols, antioxidant activity and the composition of elements, were analyzed, and the best culture conditions, such as seawater temperature and nutrient concentrations for the sporophytic and gametophytic phases, were determined. In that way, we evaluated the best fit of the abiotic conditions for their growth, controlling the fertility on the gametophyte and their long-term storage. The biochemical composition of the *L. marina* sporophyte varied according to the extraction method used. *L. marina* showed a relatively low carbohydrate and protein content, but a high polyphenol content and antioxidant activity. In the culture experiments, zygote and spore densities were significantly influenced by nutrient concentrations. The best survival conditions for gametophytes and sporophytes were at 8 °C and at 1 PES; however the growth of the sporophytes was greater than that of the gametophytes. The gametophytes could be maintained in latency under controlled conditions for 18 months. Gametogenesis suppression was observed at 8 °C in a 12:12 h L/D regime. In this study, we emphasize the need to know about both the biochemical composition of *L. marina* for industrial use and the optimal conditions for *L. marina* culture in order to establish an adequate controlled population management.

1. Introduction

Macroalgae are biologically important elements of worldwide marine systems for ecosystem functioning, aquaculture, and downstream industries [1]. Consumption of seaweeds has been reported for over three thousand years, predominantly in Asia where seaweeds are prized for their nutritional and health benefits [2]. In addition, the recent interest in culturing seaweed as food, fertilizers, biofuel, and bioactive compounds is well known [3].

Several studies have focused on the nutritional value of seaweeds through the biochemical content in order to explore their possible use as sources of protein, carbohydrate, lipid, fatty acid, minerals and pigments [4–6]. Also, many benefits of seaweed extracts exist because they contain bioactive substances like polyphenols with anti-bacterial, anti-fungal and anti-viral properties [7]. However, they are still underexploited and have not reached their full application potential [3].

Among brown algae, the *Leathesia* species are known to be sources of secondary metabolites. The presence of high contents of bromophenols [8,9], phlorotannins [10] and sulphated polysaccharides [11] have been recorded in different species. These compounds have antitumor and antioxidant activity and have also proved to be selective antiviral agents against herpes simplex virus (HSV) types 1 and 2 and human cytomegalovirus. Moreover, the extracts of *Leathesia* have shown to have in vitro trypanocidal, leishmanicidal and antimycobacterial activities [12].

Leathesia marina (Lyngbye) Decaisne has a typical heteromorphic life cycle, alternating between microscopic branched filaments (gametophyte phase) and macroscopic erect stages (sporophyte phase) [13,14]. Seaweeds with heteromorphic life cycles exhibit a high degree of independence and differentiation between the different phases. Each

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phase has unique ecological and evolutionary constraints and must be able to survive and/or reproduce under different resource and environmental conditions [15].

Many seaweed species rely on microscopic stages to survive periods of environmental stress and they produce new macroscopic stages when conditions improve [16,17]. For example, microscopic gametophytes of species of Laminariales can divide and grow under suboptimal conditions, being able to survive 16–20 months in complete darkness, and they start to reproduce when optimal conditions return [18,19].

The study of the survival of early post-settlement phases of spores and zygotes of macroalgae is critical for the successful establishment of algal populations. Abiotic variables, such as seawater temperature, radiation and nutrient concentrations, affect settlement and recruitment and therefore the dynamics of natural populations of algae [20]. Moreover, early mortality post-settlement could be the bottleneck in successful recruitment for many algae [21,22]. The adjustment of growing conditions for *Leathesia* cultures would be fundamental in providing macroalgal biomass for biotechnological purposes, avoiding the overexploitation of natural populations. Despite the potential of *L. marina* in applied phycology fields, currently there are no published data on culturing methods and optimal culture conditions.

In the present study, the biochemical composition of the *L. marina* sporophyte was analyzed in order to assess its potential use as a source of bioactive compounds. Hence, experiments were carried out to determine the organic matter, alginate, total carbohydrate and protein content, polyphenols, antioxidant activity and element composition. Moreover, the best controlled conditions for its cultivation were established, evaluating the effects of three variables: temperature, nutrient concentrations and incubation time for both the sporophytic and gametophytic phases. The culture experiments were performed in order to identify the best conditions for (i) growth, survival and storage of sporophytes and gametophytes and (ii) fertility control of gametophytes and their long-term storage. This study brings important findings that are helpful in speeding up the life cycle and providing knowledge for the possible use of *L. marina* as new, novel food and antioxidant sources.

2. Materials and methods

Leathesia marina samples were collected on the east coast of Nuevo Gulf, located on the Patagonian coast (42°46′32.57″S–62°59′23.49″W). In this temperate Patagonian zone, *L. marina* has a heteromorphic life cycle alternating between a diploid sporophytic phase and a haploid gametophytic phase (Fig. 1). The sporophytes are found at the lower intertidal zone during the summer months, whereas the gametophytes are found in the winter months [23].

2.1. Chemical composition and antioxidant activity of L. marina

The chemical analysis was performed on the macroscopic sporophyte phase, as it represents the highest biomass in respect to the microscopic phase. All determinations were performed in triplicate. First, 50 g of fresh alga were oven-dried to constant weight at 60 °C to determine the dry weight; then they were burnt at 500 °C in an electric furnace to determine the ash weight. The percentage of organic matter was calculated by subtracting the ash weight from the algal dry weight.

For total carbohydrate estimation, two extractions were tested following the method proposed by Andrade et al. [24]. 250 mg of freeze-dried alga was ground and immersed in 15 mL of Milli-Q water and stirred for 24 h. Then it was centrifuged at 21 °C (10,000*g*) for 20 min. Another sample of 250 mg was immersed in 15 mL of 5% KOH and stirred for 24 h at room temperature. After that, the algal material was separated from the supernatant by centrifugation (4 °C, 10,000*g* for 20 min). Total carbohydrates were quantified in both extracts following the method of Dubois et al. [25].

The pretreatment and extraction procedures for quantification of the alginate were based on McHugh [26]. 5 g of dried, ground and sieved algal sample was mixed with 160 mL of formaldehyde solution (0.4% w/w) under constant stirring for 30 min. The solutions were drained off, and the algae were washed with distilled water. Then, the algae were put in contact with 160 mL 0.1 N HCl for 2 h. These washing treatments are necessary to remove phenolic compounds and clarify the biomass [27]. The extraction was conducted with 110 mL 2% Na_2CO_3 for 5 h at 60 °C. The viscous mixture was filtered to separate the solid residue and the alginate solution. The alginate was precipitated with ethanol (1:1 v/v) and dried at 60 °C. The solid residue was washed exhaustively with deionized water and then dried as well. For the second extraction, the residue was again kept in contact with 160 mL 2% Na_2CO_3 for 5 h at 60 °C and the alginate precipitation was again accomplished. The alginate yields were calculated as a weight percentage (% w/w), based on the initial dry weight of the alga.

The total protein extraction procedure was based on Barbarino and Lourenco [28]. 50 mg of ground freeze-dried algal sample was immersed in 4 mL of Milli-Q water for 12 h at 4 °C. After the incubation period, it was centrifuged at 4 °C, 10,000*g* for 20 min, the supernatant was collected and kept at 4 °C and the pellet was re-extracted with 2.0 mL of 0.1 N NaOH for 1 h. Then, it was centrifuged at 4 °C, 10,000*g* for 20 min. The second supernatant was combined with the first one and the pellet was discarded. Aliquots from these extracts were used to perform the protein quantification by the Lowry method [29]. A part of the extracts obtained previously was also used for protein precipitation following Berges et al. [30]. Cold 25% trichloroacetic acid (TCA) was added in a proportion of 2.5:1 (TCA:supernatant). Tubes containing TCA and protein were incubated in an ice bath for 30 min and then centrifuged for 20 min at 4 °C (10,000*g*). The supernatant was discarded and the pellet was washed with cold 10% TCA and then centrifuged again. The pellet that formed after the second centrifugation was suspended in 5% TCA at room temperature, in a proportion of 5:1 (5% TCA:precipitate) and centrifuged at 21 °C (10,000*g*) for 20 min. The pellets were stored at −20 °C until further analysis. Precipitated protein was resuspended in 2.0 mL 0.1 N NaOH and quantified by the Lowry assays.

Two extraction solvents were tested for pigment estimation, namely 80% acetone and DMSO, based on Vimala and Poonghuzhali [31]. Chlorophyll *a* and total chlorophyll content were calculated in mg/g dry alga using the equations derived by Arnon [32] as follows:

$$\text{Chlorophyll } a\ (\mu g\ mL^{-1}) = [12.7\ (A_{663}) - 2.69\ (A_{645})]$$

$$\text{Total chlorophyll } (\mu g\ mL^{-1}) = [20.2\ (A_{645}) + 8.02\ (A_{663})]$$

Chlorophyll *c1* + *c2* content was determined using the following equation as *per* Jeffrey and Humphrey [33]:

$$\text{Chlorophyll } c1 + c2\ (mg\ g^{-1}) = [24.36\ (A_{630}) + 3.73\ (A_{664})]$$

The amount of carotenoid was estimated by the method of Kirk and Allen [34]:

$$\text{Carotenoids } (mg\ g^{-1}) = [A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645})]$$

The amount of fucoxanthin was estimated using the equation of Seely et al. [35]:

$$\text{Fucoxanthin } (mg\ g^{-1}) = [A_{470} - 1.239\ (A_{631} + A_{581} - 0.3 \times A_{664}) - 0.0275(A_{664})]/141$$

where A = Absorbance at a particular wavelength, V = Total volume of the pigment extract, W = Weight of the sample used for extraction.

Total phenolic content of the methanolic extracts (3 g of freeze-dried algal sample in 10 mL of 80% methanol) and the aqueous extracts (3 g of freeze-dried algal sample in 17.5 mL of Milli-Q water), were tested spectrophotometrically at 760 nm using the Folin-Ciocalteu method [36]. A standard curve with gallic acid solution ranging from 10 to 280 μg mL^{-1} was used for calibration. The total phenolic content

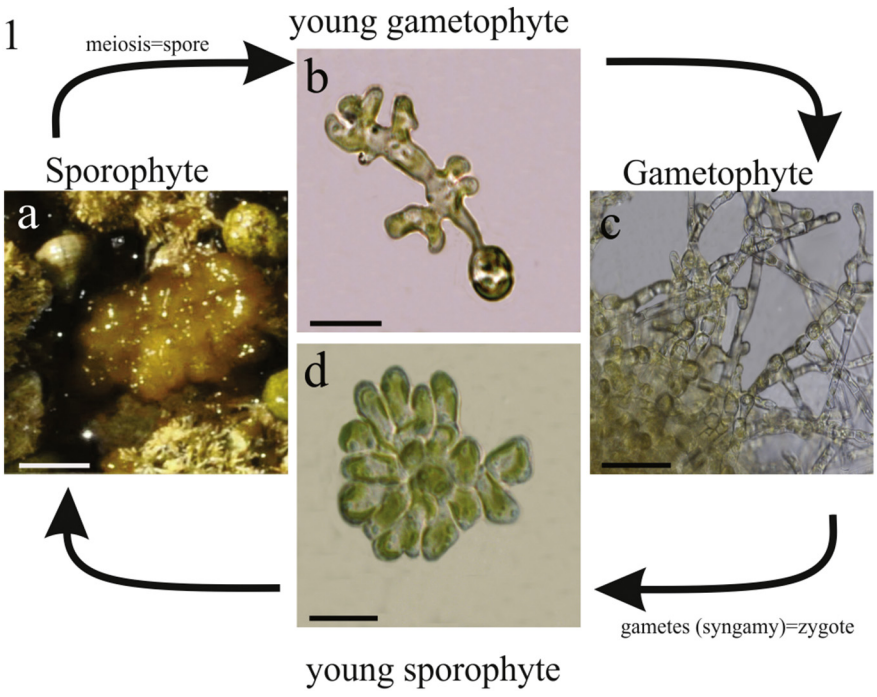

Fig. 1. Sporophyte and gametophyte phases of the heteromorphic life cycle of *L. marina*. a. Macrothalli or sporophytic phase in nature. Scale bar = 1 cm. b. young gametophyte born of a mature sporophyte. Scale bar = 10 μm. c. Microthalli or gametophytic phase in culture. Scale bar = 30 μm. d. young sporophyte born of a mature gametophyte. Scale bar = 10 μm.

was expressed as μg gallic acid equivalents per mg of dry alga (μg GAE mg^{-1} dry alga).

The antioxidant activity of methanolic extract was estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The reaction mixture was prepared by adding 250 μL of extract to 3 mL of methanolic solution of DPPH radical with an absorbance equal to 1 at 517 nm [37]. The mixture was stirred and allowed to stand for 30 min at room temperature in the dark. The absorbance of the resulting solution was measured at 517 nm. The percentage inhibition of the DPPH radical of the samples was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = 100x\left[1 - \left(\frac{\text{Asample} - \text{Ablank}}{\text{Acontrol}}\right)\right]$$

where Acontrol is the absorbance of the control (DPPH solution without sample), the Asample is the absorbance of the test sample (DPPH solution plus test sample), and the Ablank is only the absorbance of the sample (sample without DPPH solution). The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against the extract concentrations. The total antioxidant activity was expressed as the vitamin C equivalent antioxidant capacity (VCEAC).

To determine the presence of different elements on sporophytes of *L. marina*, fragments of the thallus were fixed in 2.5% glutaraldehyde containing 0.01 M sodium cacodilate (pH 7.2) buffer at 5 °C for 2 h. Subsequently, three washes in buffer sodium cacodylate 0.005 M were performed for 10 min each. The fixed portions were dehydrated in acetone following the protocol by Cáceres [38]. Finally, the samples were dried at critical-point for 1 h and coated with gold in a Sputter Coater 9100 mod.3 according to Sorrivas de Lozano and Morales [39]. Samples were observed under a scanning electron microscope (SEM) Leo Evo 40 (Jena, Germany) equipped with the OXFORD X- Max^{50} Energy Dispersive X-ray microanalysis (EDX) system (Oxford Instruments, Abingdon, Oxfordshire, UK).

2.2. Culture assays of L. marina

The gametophytes were obtained from a culture of spores formed in reproductive structures on sporophytes collected in the wild. Previously, these sporophytes were brushed and rinsed with sterile filtered (0.2 μm) seawater to remove epiphytes and sediment.

The sporophytes were obtained from zygotes from stock gametophytic cultures (C.15). These stocks had been maintained in seawater since 2015 under controlled temperature and nutrient conditions, i.e. 8 °C and 2 mL·L^{-1} Provasoli culture medium (PES).

All culture experiments were performed in 2.5 cm diameter Petri dishes with PES, containing 2 mm^2-fertile fragments of sporophytes or gametophytes on a 1.5 cm^2-coverslip. Attached zygote and spore development was monitored on the coverslips. The light (L)/dark (D) regime was 12:12 h with an irradiance of 25 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes. The irradiance was monitored using a Quantum Flux meter (Apogee MQ-200, USA).

To determine the optimal growth and survival of spores and zygotes, a factorial experiment was carried out on the sporophytic and gametophytic phases to evaluate four nutrient concentrations and three incubation temperatures. In order to evaluate the effect of nutrient concentrations on survival and growth of spores and zygotes, PES concentrations added to the seawater were drastically reduced or increased. Four nutrient concentrations, namely 0, 0.1, 1 and 10 times the concentrations suggested by Oliveira et al. [40] (2 mL PES/L), were evaluated during the 10-day incubation period. The incubation at each nutrient concentration was performed by triplicate and was exposed at 8 °C, 16 °C and 24 °C. These two variables were evaluated every two days over a period of 10 days of incubation, considering the time as a third variable. The culture medium was refreshed every two days in all cases. In previous culture experiences the formation of reproductive structures was observed after 15 days of incubation, and so in all cases the time of experimentation did not exceed 10 days so as to avoid the formation of gametangia and sporangia.

Thalli survival was measured by counting the live individuals in an area of 3 cm^2 using a Nikon Eclipse TE 300 microscope (Tokyo, Japan) equipped with a Nikon FDX 35 camera. Counts were conducted using 400× total magnification. The vegetative growth was measured by counting the number of germling cells. The measurements of survival and vegetative growth were performed every two days.

2.2.1. Fertility control on gametophyte

In order to test the gametogenesis, gametophytes were cultivated in seawater enriched with PES (2 mL PES/L) at 8 °C, 16 °C and 24 °C with

12:12 h L/D and an irradiance of 25 μmol $m^{-2} s^{-1}$ provided by cool white fluorescent tubes. Plurilocular gametangia were counted in fragments of 2 mm^2 from 20 gametophytes from each incubation temperature. Counts were performed using a light microscope at 400× magnification.

2.3. Statistical analyses

To evaluate the difference in chemical composition of *L. marina* depending on the solvent of extraction, a Student's *t*-test was used (unpaired two-sample t-test). The statistical program R Studio (Team 2016) was used at a 0.05 significance level.

For evaluation of the settled spore and zygote density (n° spores/zygotes/3 cm^2), a generalized linear model with a binomial negative error distribution (GLM.nb) was used as a traditional method for handling overdispersed Poisson. The analyses were performed using a "MASS" package in R [41].

For survival analysis, series of generalized linear mixed models (GLMM) with a binomial error distribution were used. All GLMMs were performed with "lme4" package in R [42]. For the evaluation of survival in relation to incubation times, temperatures and nutrient concentrations, additive and interaction models were considered. The model was selected according to the Akaike's information criterion (AIC), where the smaller AIC is the model that best fits the data. In this case, the additive model had the lower AIC.

For fertility analysis, Zero-inflated Poisson (ZIP) was used because of most of the gametophytes incubated at 16 °C did not have any gametangia. The analyses were performed using a "pscl" package in R [43].

The vegetative growth was analyzed with linear mixed-effects models since the light was plentiful during the experiment and the nutrients were periodically replenished, so the absolute growth rate was not expected to slow down during the study period. The additive and interaction models were tested. The additive model had the lower AIC. The linear mixed-effects models were determined using the maximum likelihood (ML) estimation with a "nlme" package in R [44]; the varPower function to control the heteroscedasticity effect and goodness-of-fit with *Pseudo*-R^2 for models was estimated with a "piecewiseSEM" package in R [45].

In all the analyzes, significant differences between treatments are indicated by different letters using Tukey's Honest Significant Difference test (HSD) using a "multcomp" package in R [46].

3. Results

3.1. Biochemical properties and antioxidant activity of L. marina

L. marina showed carbohydrate contents of 19.9 ± 2.4 and 16.9 ± 2.3 mg (mean ± SE) of carbohydrate/100 mg of organic matter for KOH and aqueous extract, respectively. No significant differences were observed in the carbohydrate content between the two extraction types (Table 1). The content of alginate was 15.55 ± 1.10%. Protein content in crude extracts showed higher values than those obtained from precipitated samples, being 1.01 ± 0.06% (Table 1).

The main photosynthetic pigments, i.e. chlorophyll, carotenoid and fucoxanthin, presented a better extraction with 80% acetone than DMSO; except for chlorophyll *c1* + *c2*, which presented no significant differences between the two types of extraction (Table 1). Chlorophyll *a* (chl *a*) was the most abundant pigment, followed by carotene (Cart) and chlorophyll *c1* + *c2* (Chl *c1* + *c2*). The pigment recorded at the lowest concentration was fucoxanthin.

The highest level of phenolic content was observed in aqueous extract with 0.99 ± 0.04 mg GAE/g (Table 1). It was only possible to measure the DPPH radical scavenging activity in the methanolic extract because the aqueous extract in contact with DPPH -methanol solution formed a precipitate. The extract concentration required to reduce the initial DPPH concentration by 50% (IC_{50}) was 16.9 mg/mL. And the radical scavenging activity expressed as vitamin C equivalent was 17.44 ± 0.61 mg VCE/100 g of dry algae.

Table 1
Biochemical characteristics of *L. marina* sporophyte (percentage of dry weight, ash, organic matter, total carbohydrate, alginate, protein, pigment, polyphenol contents) and antioxidant activity (DPPH radical scavenging activity). Data represent the mean of three replicates ± S.E. (n = 3). Student's *t*-test was used to evaluate the difference in chemical composition of *L. marina* depending on the solvent of extraction; *df*, degrees of freedom; *p-value* (< 0.05), probability. Abbreviation: Chlorophyll (Chl), carotenoids (Cart), dry sample (DS).

Biochemical characteristics	Solvent	*Mean* ± *SE*	Statistic (*t*)	*df*	*p-value*
Dry weight (%)		70.53 ± 0.69			
Ash		41.79 ± 1.38			
Organic matter (%)		29.46 ± 0.69			
Total carbohydrates (mg/100 mg organic matter)	Water	16.9 ± 2.3	-0.89	4	0.4192
	KOH extract	19.9 ± 2.4			
Alginate (%)		15.55 ± 1.10			
Proteins (%)	Water	1.01 ± 0.06	3.70	4	0.0207
	Precipitated with TCA	0.53 ± 0.12			
Pigment					
Chl *a* (mg/g DS)	DMSO	0.14 ± 0.008	21.63	4	< 0.0001
	80% acetone	0.46 ± 0.013			
Total Chl *a* + *b* (mg/g DS)	DMSO	0.16 ± 0.009	23.42	4	< 0.0001
	80% acetone	0.53 ± 0.014			
Chl *c1* + *c2* (mg/g DS)	DMSO	0.11 ± 0.016			
	80% acetone	0.17 ± 0.019	2.48	4	0.0693
Cart (mg/g DS)	DMSO	0.15 ± 0.009	26.40	4	< 0.0001
	80% acetone	0.36 ± 0.005			
Fucoxanthin (mg/g DS)	DMSO	0.002 ± 0.001	28.06	4	< 0.0001
	80% acetone	0.006 ± 0.001			
Total polyphenols (mg GAE/g DS)	Water	0.99 ± 0.04	10.61	4	0.0004
	80% methanol	0.51 ± 0.02			
Radical scavenging IC_{50} (mg/mL)	80% methanol	16.96 ± 0.66			
VCEAC (mg VCE/100 g DS)	80% methanol	17.44 ± 0.61			

The EDX spectra revealed the presence of different elements, such as Cu, Na, Ca, S, Mg, and Si, on the external surface of the *L. marina* cell walls. Al and Fe were found in very low proportion. The presence of gold corresponds to the gold coating, necessary for observation in a scanning electron microscope (SEM) (Fig. 2).

3.2. Culture assays of L. marina

3.2.1. Spores and zygote settlement

Gametes developed on gametophytes were released after 24 h of incubation and they settled approximately 48 h after the fusion. However, the spores developed on sporophytes were released after 12 h of incubation and settled after 36 h, indicating a faster recruitment of the sporophytic phase. The densities of both zygotes and spores were significantly influenced by the nutrient concentrations (Table 2). However, the temperature had no influence on zygote and spore settlement. At all temperature and nutrient concentrations, the number of spores settled (44 ± 9 spores) was higher than the number of zygotes settled (21 ± 2 zygotes). The highest numbers of spores settled were recorded at concentration 1 PES (52 ± 25 spores) and the zygotes at

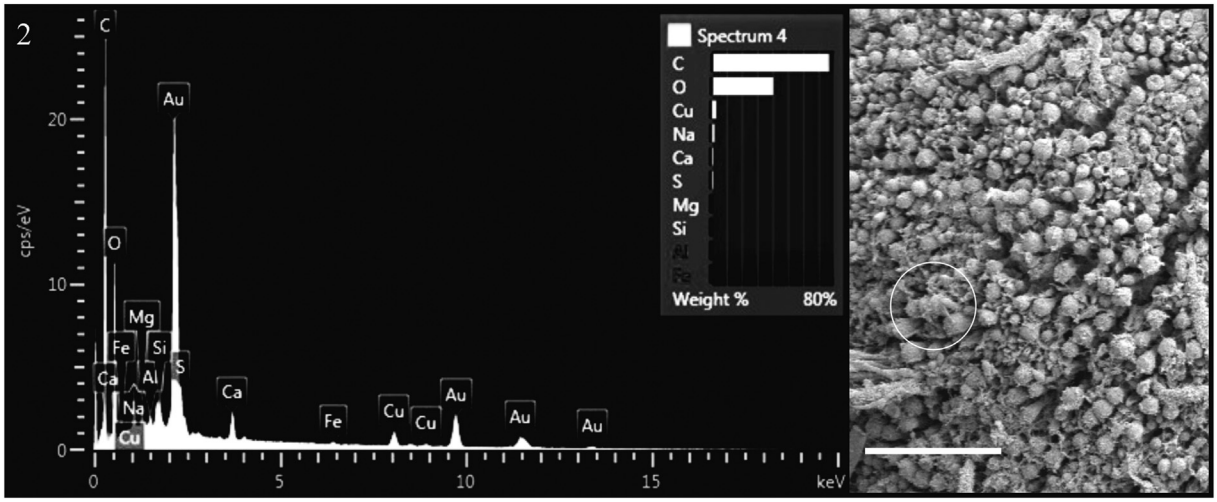

Fig. 2. SEM-EDX analysis of sporophyte of *L. marina*: external surface. Scale bar = 100 μm.

Table 2
Summary statistics GLM.nb analysis testing the temperature and nutrient concentrations on density of settled spores and zygotes (n° spores/zygotes/3 cm^2), and relationship between both zygote and spore densities. GLMM analysis testing the effects of incubation time, temperature and nutrient concentrations on survival in sporophyte and gametophyte of *L. marina* and the relationship between survival of sporophytes and gametophytes. ZIP analysis testing the effects of incubation temperature on plurilocular gametangia number. *df*, degrees of freedom, *p* values (< 0.05), probability.

	df	Statistic (χ^2)	*p-value*
GLM.nb			
Zygotes vs spores	1	15.21	< 0.0001
N° spores			
Nutrient concentrations	3	8.32	0.039
Temperature	2	0.52	0.7684
N° zygotes			
Nutrient concentrations	3	16.42	< 0.0001
Temperature	2	2.31	0.3165
GLMM			
Sporophyte vs gametophyte survival	1	7.45	0.006
Sporophyte survival			
Time	4	1560.7	< 0.0001
Temperature	2	45.471	< 0.0001
Nutrient concentrations	3	0.1406	0.9866
Time + Temperature	6	444.81	< 0.0001
Gametophyte survival			
Time	4	606.52	< 0.0001
Temperature	2	45.471	< 0.0001
Nutrient concentrations	3	19.009	< 0.0001
Time + Temperature + Nutrient	9	766.35	< 0.0001
ZIP: Microthalli stage.			
Plurilocular gametangia number			
Temperature	1	76.92	< 0.0001

10 PES (31 ± 6 zygotes). The lowest numbers of zygotes settled were obtained in cultures free of PES (12 ± 2 zygotes) and the spores at a concentration of 0.1 PES (14 ± 5 spores) (Fig. 3).

3.2.2. Survival of sporophytes and gametophytes

Significant differences were observed in the survival of sporophytes and gametophytes; i.e. 42.4 ± 7.82% and 52.14 ± 7.19%, respectively (Table 2).

The survival of sporophytes was influenced by incubation time and seawater temperature, whereas, the effect of nutrient concentrations was not significant (Table 2). However, the survival of gametophytes was affected by incubation time, temperature, and nutrient concentrations.

The variable incubation time affected the survival of both sporophytes and gametophytes negatively since a decreased survival was observed as the incubation time progressed. The maximum survival of both sporophytes and gametophytes was observed after 2 days of incubation (reaching values of 90.9 ± 1.2% and 98.2 ± 10.3%, respectively) and the minimum after 10 days, showing a survival of 48.22 ± 4.2% and 52.25 ± 6.27%, respectively. An abrupt decline in survival was observed at the beginning of the fourth day of experimentation (Fig. 4). At each time of experimentation evaluated, the gametophyte survival was greater than the sporophytes (Fig. 4).

The optimal temperature for survival of the sporophytes and gametophytes was 8 °C, reaching values of survival of 51.74 ± 5.10% and 58.2 ± 6.3%, respectively. The lowest survivals for both sporophytes and gametophytes were recorded at temperatures of 16 °C, i.e.43.76 ± 3.53% and 44.2 ± 6.14, respectively and 24 °C, i.e. 39.93 ± 2.07%, 42 ± 9.28%, respectively (Fig. 5).

The different nutrient concentrations showed significant differences on gametophyte survival (Table 2). The highest survival was recorded at concentration 1 (PES) with 64.9 ± 4.7% and the lowest survival was seen in the cultures free of PES, with 37.81 ± 5.64% (Fig. 6).

3.2.3. Growth of sporophytes and gametophytes

The absolute growth rate for sporophytes at different temperatures and nutrient concentrations is shown in Table 3. Both temperature and nutrient concentration variables had a significant effect on vegetative growth. The sporophytes growing at 24 °C showed faster growth, while the sporophytes incubated at 8 °C had a lower growth rate. The highest growth was recorded at concentrations of 1 PES and 10 PES, while the lowest growth was observed in the cultures without any PES (Table 3).

The absolute growth rate for gametophytes at different temperatures and nutrient concentrations is also shown in Table 3. Both the temperature and nutrient concentration variables had significant effects on the growth rate. The thalli incubated at 8 °C and 16 °C had a faster growth rate than the gametophytes incubated at 24 °C·The gametophytes incubated at 1 PES and 10 PES concentrations had the highest growth, while the lowest growth was observed in the cultures without any PES and 0.1 PES (Table 3).

Significant differences in growth were observed for both gametophytes and sporophytes; the sporophytes reached a higher growth rate than the gametophytes (Table 3).

3.2.4. Maintenance of gametophytes under controlled culture conditions

Incubation time and the seawater temperature affected the gametogenesis. Therefore, these microscopic prostrate gametophytes were maintained in latency under controlled culture conditions for a long

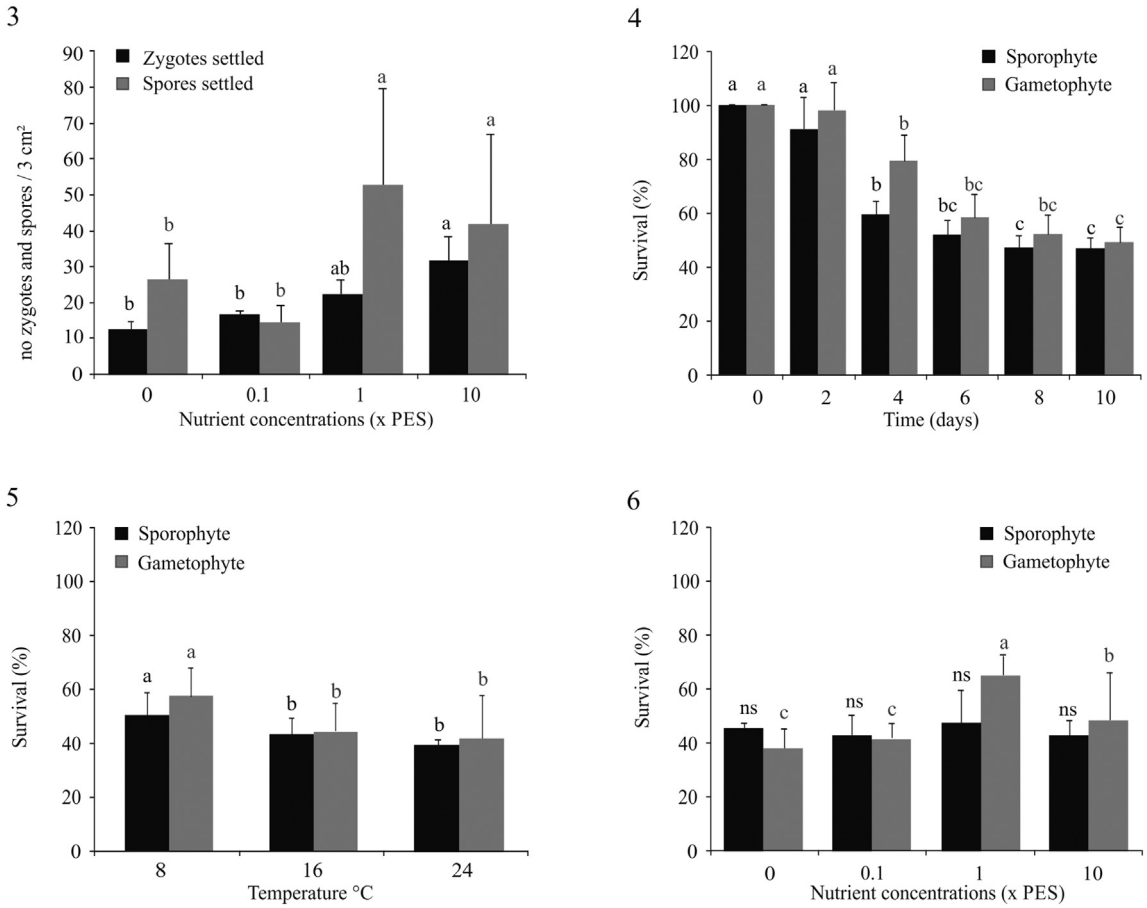

Fig. 3–6. Culture assays of *L. marina*. **Fig. 3**. Effect of nutrient concentration on density of zygotes and spores settled. Error bars show standard errors. **Fig. 4**. Effect of incubation time on the survival of sporophytes and gametophytes. **Fig. 5**. Effect of incubation temperature on the survival of sporophytes and gametophytes. **Fig. 6**. Effect of nutrient concentration on the survival of sporophytes and gametophytes. Significant differences ($\alpha < 0.05$) are indicated by different letters using Tukey's Honestly Significant Difference (HSD). Letter in black compares zygotes settled/sporophyte and letter in gray compares spores settled/ gametophyte.

time. Suppression of the gametogenesis process was observed at 8 °C, in12:12 h L/D and a concentration of 2 ml PES/L. Under this condition, the gametophytes were maintained for 18 months with weekly replacement of the culture medium without any formation of plurilocular gametangia. Nevertheless, the gametogenesis process was stimulated by raising the temperature to 16 °C and 24 °C. Under these new conditions a clear formation and development of gametangia was observed. After 20 incubation days at both temperatures, significant differences in the number of plurilocular gametangia were observed, i.e. 25.4 ± 12.23 gametangia on 58.74 mm^2 of microthallus were formed at 24 °C, while at 16 °C, the number of gametangia was significantly lower and only 2.3 ± 1.15 on 58.74 mm^2 of microthallus.

4. Discussion

Seaweeds, considered as a food source for their nutritional value, have also been linked to the prevention and/or treatment of different diseases related to oxidative stress, an aspect that has served as a guideline for research into its antioxidant properties [47–49]. For this reason, the biochemical properties of *L. marina* were analyzed using different extraction methods and solvents. Sporophytes of *L. marina* from Patagonian coasts showed a low organic matter percentage and total carbohydrate content in comparison to other brown algae, e.g. *Cystoseira trinodis* (Forsskål) C·Agardh, *Sargassum dentifolium* (Turner) C. Agardh and *Sargassum asperifolium* Hering & G. Martens ex J. Agardh [50]. However, *Macrocystis pyrifera* (L) C. Agardh and *Undaria pinnatifida* (Harvey) Seringar, which also inhabit the Patagonian coasts and which showed a total carbohydrate content similar to that found in *L. marina* [5].

Algins/alginates extracted from brown seaweed are dietary polysaccharides and are not found in any land plants. These are available in both acid and salt forms. It has been reported in the literature that the alginates lead to a decrease in the concentration of cholesterol, exert an anti-hypertension effect, can prevent absorption of toxic chemical substances, and play a major role as dietary fiber for the maintenance of animal and human health [51,52]. The alginate yield determined in this study was similar to those found for *M. pyrifera* and *U. pinnatifida* from the Patagonian coasts, ranging between 10 and 30% dry weight [5]. Seaweed protein content differs according to the species and phyla, also depending on factors such as season, geographic distribution, seawater temperature, nutrient concentrations, salinity, harvesting time and even the population [53,54]. Brown seaweed protein content is generally small, with an average of 3–15% dry weight [55]. The value found in *L. marina* is lower than the average; however, it was similar to the brown alga *U. pinnatifida* from the Patagonian coasts [5]. Mabeau and Fleurence [56] suggest that the high phenolic content in brown seaweed species might limit protein availability in vivo and thus moderate in vitro. This limitation is not found in the green and red seaweeds, which possess low levels of phenols and higher protein contents. For example, a protein content of 35% dry matter was reported for *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr and 44% dry matter for *Ulva* spp. [57]. Concerning the pigment content in chlorophylls, chlorophyll *a* (chl a) is a major pigment in *L. marina*. Chlorophyll *a* is essential in the reaction centre of the thylakoid, light-harvesting structures in which photosynthesis is carried out [58,59]. It is known that Chlorophyll is converted into pheophytin,

Table 3
Statistical data for vegetative growth of sporophyte and gametophyte of *L. marina* were analyzed with linear mixed-effects models, contrasting the slopes for the treatments of temperature and concentration of nutrients. The lineal model was determined using maximum likelihood (ML), varPower function to control the heteroscedasticity and a goodness-of-fit statistics for linear mixed-effects models (*Pseudo*-R^2). *df*, degrees of freedom; *p*-value (< 0.05), probability. Significant differences between treatments are indicated by different letters using Tukey's Honestly Significant Difference (HSD).

Sporophyte					
Model: N°cell~Time:Temperature + Time:Nutrient concentrations (*Pseudo*-R^2 = 0.99)					
Temperature (°C)	Growth rate (mean ± SE)	*df*	Statistic (*F*)	*p-value*	HSD
8	1.12 ± 0.09	162	18.58	< 0.0001	b
16	1.46 ± 0.13				a
24	1.77 ± 0.13				a
Nutrient concentrations					
0	1.16 ± 0.10	162	176.81	< 0.0001	b
0.1	1.31 ± 0.11				ab
1	1.58 ± 0.14				a
10	1.75 ± 0.15				a
Gametophyte					
Model: N°cell~Time:Temperature + Time:Nutrient concentrations (*Pseudo*-R^2 = 0.98)					
Temperature (°C)	Growth rate (mean ± SE)	*df*	Statistic (*F*)	*p-value*	HSD
8	0.92 ± 0.06	162	16.72	< 0.0001	a
16	0.86 ± 0.07				a
24	0.61 ± 0.05				b
Nutrient concentrations					
0	0.70 ± 0.23	162	161.66	< 0.0001	b
0.1	0.72 ± 0.36				b
1	0.99 ± 0.27				a
10	0.89 ± 0.39				a
Comparison between gametophytic vs sporophytic stage					
	Growth rate (mean ± SE)	*df*	Statistic (*F*)	*p-value*	HSD
Sporophyte	1.36 ± 0.07	346	191.84	< 0.0001	a
Gametophyte	0.90 ± 0.05				b

pyropheophytin and pheophorbide in processed vegetable food and following ingestion by humans. These derivates show antimutagenic effects and may play a significant role in cancer prevention [60]. The brown seaweeds are particularly rich in carotenoids that are characterized as powerful antioxidants, preventing human pathologies linked to oxidative stress [61,62].

L. marina showed a content of carotenoids similar to *Padina gymnospora* (Kützing) Sonder [63], and an average content of fucoxanthins similar to those found in *Sargassum polycystum* C.Agardh, *Turbinaria conoides* (J.Agardh) Kützing and *Hydroclathrus clathratus* (C.Agardh) M.Howe [31].

Algal polyphenols have been highly regarded for their important dietary roles as antioxidants and chemopreventive agents, as antimicrobial and antiviral activities [64]. *L. marina* showed a high total phenolic content in the aqueous extract, similar to other brown algae, such as *Desmarestia confervoides* (Bory) M.E. Ramírez & A.F Peters (previously known as *Desmarestia viridis*) and *Dictyopteris divaricata* (Okamura) Okamura [65]. In relation to the DPPH radical scavenging assay, the sample concentration necessary for providing 50% inhibition DPPH was 16 mg/mL and the radical scavenging activity expressed as vitamin C equivalent was 17.44 ± 0.61 mg VCE/100 g of dry algae. Similar 50% inhibition DPPH was reported in extract concentrations of *Dictyota ciliolata* Sonder ex Kützing [66].

Several studies on brown algae population dynamics have focused on processes regulating survival, growth and the fertility of different life cycle stages [67–69]. Abiotic factors, such as seawater temperature, nutrient concentrations and light requirements, are known as regulators of seaweed development and phenology [70–72]. In this study, we analyse how abiotic factors, such as temperature and nutrient concentrations, affect the development of *L. marina* under culture condition. These studies are fundamental for establishing optimal conditions for controlled cultures, whose regulation is essential for the management of the *L. marina* life cycle.

Early post-settlement of spore and zygote survival derived from the sporophytes and gametophytes was critical for the successful establishment of benthic populations. Spore and zygote settlement occurred after 24 to 48 h of incubation and their densities were favoured by high nutrient concentrations, but they were not influenced by temperature. However, seawater temperature had a clear effect on survival and growth on subsequent incubation days. These results demonstrated that *L. marina* endures a wide range of temperatures, and low nutrient concentrations limited spore and zygote settlements.

PES culture medium contains high nitrogen and phosphorus concentrations, along with micronutrients and vitamins. In temperate coastal areas, it is assumed that inorganic N and P availability is the main control for macroalgal growth [73–76]. Amsler and Neushul [77] found that brown algae motile spores, such as *Macrocystis pyrifera* and *Pterygophora californica* Ruprecht, responded to a variety of inorganic and organic nutrients, where the spore chemotaxis can actively detect microhabitats that are nutritionally favourable for their development. This could explain the higher density and survival of spores and zygotes in treatments with higher concentration of nutrients.

Even though the germlings of *L. marina* survived at the three temperatures tested (8 °C, 16 °C and 24 °C), the optimal survival

temperature for spores and zygotes was the lowest one. Macroalgal species of temperate climates with heteromorphic life cycles, such as *L. marina*, exhibit a broad "performance breadth" and typically grow in environments with large fluctuations in seawater temperature. The tolerance of *L. marina* to low and high temperatures could lead us to consider it a eurythermal species [18]. Eurythermal seaweed growth typically correlates with the local temperature regime [78]. Here, we have found differences in optimal growth for sporophytes and gametophytes. The sporophytes not only grew faster than gametophytes, but they also presented a different optimal temperature. The highest growth of sporophytes was at warm temperatures, whereas the highest growth of gametophytes was at cold ones. This was also observed in *Myriotrichia clavaeformis* Harvey which has a winter gametophytic phase and a summer sporophytic phase [79]. This reflects the capacity of heteromorphic life phases to exploit different ecological niches by being adapted to environments that differ in terms of temperature, light levels and nutrient concentrations [80–82]. Other species, such as *Ectocarpus crouaniorum* Thuret and *Ectocarpus siliculosus* (Dillwyn) Lyngbye, also present gametophytes and sporophytes that occupied different spatio-temporal niches. The gametophytes were found as epiphytes on the alga *Scytosiphon lomentaria* (Lyngbye) Link during spring whereas sporophytes were present all year-round on abiotic substrata [83]. Therefore, the optimum tolerance for the algal populations in the wild is influenced by the local conditions where they live. On the Patagonian coast, the macroscopic phase of *L. marina* was found during eight months corresponding to the warm season, whereas the microscopic gametophytic phase was found during colder months [23].

Temperature also affects the gametophytic phase permanence - fundamental for the sporophytic phase recruitment and hence, key in controlling variability in the populations [84]. The microscopic gametophytes of *L. marina* could be stored for a long time at 8 °C without any initiation of the gametogenesis process. The formation of reproductive structures was only initiated at high temperatures. The same behavior has also been reported in other brown algae, such as *Glossophora kunthii* (C. Agardh) J. Agardh and *Dictyota dichotoma* (Hudson) J.V. Lamouroux, where low temperatures inhibited the formation of reproductive structures [85,86].

Increases or decreases of nutrient concentrations caused differences in the growth and survival rates. According to Carney and Edwards [71], nutrient limitation generates a delay in development and inhibits reproduction. In this study, the young gametophytes and sporophytes presented lower growth rates in cultures without or with low PES, but they were favoured at high PES concentrations. However, sporophytes survival was not affected significantly by nutrient concentrations. These results demonstrate that young sporophytes were more resistant to unfavourable nutrient concentrations.

In this study, we emphasize the need to know about the biochemical composition of *L. marina* for its use in the industry and the optimal culture conditions in order to establish controlled management of its life cycle. The maintenance of gametophytes in latency under controlled conditions for a long time enables protection of the natural populations, avoiding overexploitation.

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Author contributions

Ailen M. Poza and M. Cecilia Gauna are responsible for the integrity of the work as a whole; all named authors contributed materially to the execution of the final manuscript and were involved in the inception, data acquisition, analysis and interpretation and in drafting and editing the manuscript.

Disclosure statement

We declare that all authors agree to authorship and submission of the manuscript. There is no conflict of interest with the outcomes of this research. No conflicts, informed consent, human or animal rights are applicable to this research.

References

[1] J.T. Hafting, J.S. Craigie, D.B. Stengel, R.R. Loureiro, A.H. Buschmann, C. Yarish, M.D. Edwards, A.T. Critchley, Prospects and challenges for industrial production of seaweed bioactives, J. Phycol. 51 (2015) 821–837, http://dx.doi.org/10.1111/jpy.12326.
[2] S. Kumar, D. Sahoo, I. Levine, Assessment of nutritional value in a brown seaweed *Sargassum wightii* and their seasonal variations, Algal Res. 9 (2015) 117–125, http://dx.doi.org/10.1016/j.algal.2015.02.024.
[3] P. Baweja, S. Kumar, D. Sahoo, I. Levine, Biology of seaweeds, in: J. Fleurence, I. Levine (Eds.), Seaweed in Health and Disease Prevention, Academic Press (Elsevier), Lewiston, 2016, pp. 41–106, , http://dx.doi.org/10.1016/B978-0-12-802772-1.00003-8.
[4] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation, J. Appl. Phycol. 23 (2011) 543–597, http://dx.doi.org/10.1007/s10811-010-9632-5.
[5] J.P. Cazón, M. Viera, S. Sala, E. Donati, Biochemical characterization of *Macrocystis pyrifera* and *Undaria pinnatifida* (Phaeophyceae) in relation to their potentiality as biosorbents, Phycologia 53 (2014) 100–108, http://dx.doi.org/10.2216/12-106.1.
[6] P. Verma, M. Kumar, G. Mishra, D. Sahoo, Multivariate analysis of fatty acid and biochemical constitutes of seaweeds to characterize their potential as bioresource for biofuel and fine chemicals, Bioresour. Technol. 226 (2017) 132–144, http://dx.doi.org/10.1016/j.biortech.2016.11.044.
[7] C.S. Kumar, P. Ganesan, P.V. Suresh, N. Bhaskar, Seaweeds as a source of nutritionally beneficial compounds—a review, J. Food Sci. Technol. 45 (2008) 1–13.
[8] N. Xu, X. Fan, X. Yan, C.K. Tseng, Screening marine algae from China for their antitumor activities, J. Appl. Phycol. 16 (2004) 451–456, http://dx.doi.org/10.1007/s10811-004-5508-x.
[9] D. Shi, J. Li, S. Guo, H. Su, X. Fan, The antitumor effect of bromophenol derivatives in vitro and *Leathesia nana* extract in vivo, Chin. J. Oceanol. Limnol. 27 (2009) 277–282, http://dx.doi.org/10.1007/s00343-009-9119-x.
[10] S. Heo, S. Cha, K. Lee, S.K. Cho, Y. Jeon, Antioxidant activities of Chlorophyta and Phaeophyta from Jeju Island, Algae 20 (2005) 251–260, http://dx.doi.org/10.4490/ALGAE.2005.20.3.251.
[11] S.C. Feldman, S. Reynaldi, C.A. Stortz, A.S. Cerezo, E.B. Damonte, Antiviral properties of fucoidan fractions from *Leathesia difformis*, Phytomedicine 6 (1999) 335–340, http://dx.doi.org/10.1016/S0944-7113(99)80055-5.
[12] J. Spavieri, A. Allmendinger, M. Kaiser, R. Casey, S. Hingley-Wilson, A. Lalvani, D. Tasdemir, Antimycobacterial, antiprotozoal and cytotoxic potential of twenty-one brown algae (Phaeophyceae) from British and Irish waters, Phytother. Res. 24 (2010) 1724–1729, http://dx.doi.org/10.1002/ptr.3208.
[13] P. Dangeard, Recherches sur le cycle évolutif de *Leathesia difformis* (L.) Areschoug, Botaniste 48 (1965) 5–43.
[14] A.F. Peters, Reproduction and sexuality in the Chordariales (Phaeophyceae). A review of culture studies, Prog. Phycol. Res. 5 (1987) 223–263.
[15] D.R. Schiel, M.S. Foster, The population biology of large brown seaweeds: ecological consequences of multiphase life histories in dynamic coastal environments, Annu. Rev. Ecol. Evol. Syst. 37 (2006) 343–372, http://dx.doi.org/10.1146/annurev.ecolsys.37.091305.110251.
[16] A.J. Hoffmann, B. Santelices, Banks of algal microscopic forms: hypotheses on their functioning and comparisons with seed banks, Mar. Ecol. Prog. Ser. 79 (1991) 185–194.
[17] M.S. Edwards, The role of alternate life-history stages of a marine macroalga: a seed bank analog? Ecology 81 (2000) 2404–2425, http://dx.doi.org/10.1890/0012-9658(2000)081[2404:TROALH]2.0.CO;2.
[18] I. tom Dieck, Temperature tolerance and survival in darkness of kelp gametophytes

(Laminariales, Phaeophyta): ecological and biogeographical implications, Mar. Ecol. Prog. Ser. 100 (1993) 253–264.
[19] Y. Yoneshigue-Valentin, The life cycle of *Laminaria abyssalis* (Laminariales, Phaeophyta) in culture, Hydrobiologia 204 (1990) 461–466, http://dx.doi.org/10.1007/978-94-009-2049-1_65.
[20] A.J. Underwood, P.G. Fairweather, Supply-side ecology and benthic marine assemblages, Trends Ecol. Evol. 4 (1989) 16–20.
[21] J. Mc Lachlan, Effects of temperature and light on growth of embryos of *Fucus edantatus* and *F. distichus* ssp. *distichus*, Can. J. Bot. 52 (1974) 943–951.
[22] R.L. Vadas Sr., S. Johnson, T.A. Norton, Recruitment and mortality of early post-settlement stages of benthic algae, Br. Phycol. J. 27 (1992) 331–351, http://dx.doi.org/10.1080/00071619200650291.
[23] A.M. Poza, M.C. Gauna, J.F. Escobar, E.R. Parodi, Heteromorphic phases of *Leathesia marina* (Ectocarpales, Ochrophyta) over time from northern Patagonia, Argentina, Phycologia 56 (2017) 579–589, http://dx.doi.org/10.2216/16-117.1.
[24] L.R. Andrade, L.T. Salgado, M. Farina, M.S. Pereira, P.A. Mourao, G.M. Amado Filho, Ultrastructure of acidic polysaccharides from the cell walls of brown algae, J. Struct. Biol. 145 (2004) 216–225, http://dx.doi.org/10.1016/j.jsb.2003.11.011.
[25] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[26] D.J. Mc Hugh, Production, properties and uses of alginates, Production and Utilization of Products From Commercial Seaweeds, FAO. Fish. Tech. Pap, 288 1987, pp. 58–115.
[27] C. Bertagnolli, M.G.C. da Silva, E. Guibal, Chromium biosorption using the residue of alginate extraction from *Sargassum filipendula*, Chem. Eng. J. 237 (2014) 362–371, http://dx.doi.org/10.1016/j.cej.2013.10.024.
[28] E. Barbarino, S.O. Lourenço, An evaluation of methods for extraction and quantification of protein from marine macro and microalgae, J. Appl. Phycol. 17 (2005) 447–460, http://dx.doi.org/10.1007/s10811-005-1641-4.
[29] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[30] J.A. Berges, A.E. Fischer, P.J. Harrison, A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from marine diatom *Thalassiosira pseudonana*, Mar. Biol. 115 (1993) 187–193.
[31] T. Vimala, T.V. Poonghuzhali, Estimation of pigments from seaweeds by using acetone and DMSO, Int. J. Sci. Res. 4 (2015) 1850–1854.
[32] D.I. Arnon, Copper enzymes in isolated chloroplasts, polyphenol oxidase in *Beta vulgaris*, Plant Physiol. 2 (1949) 1–15.
[33] S.T. Jeffrey, G.F. Humphrey, New spectrophotometric equations for determining chlorophylls a, b, c 1 and c 2 in higher plants, algae and natural phytoplankton, Biochem. Physiol. Pflanz. 167 (1975) 191–194, http://dx.doi.org/10.1016/S0015-3796(17)30778-3.
[34] J.T.O. Kirk, R.L. Allen, Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione, Biochem. Biophys. Res. Commun. 21 (1965) 523–530.
[35] G.R. Seely, M.J. Duncan, W.E. Vidaver, Preparative and analytical extraction of pigments from brown algae with dimethyl sulfoxide, Mar. Biol. 12 (1972) 184–188, http://dx.doi.org/10.1007/BF00350754.
[36] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, Methods Enzymol. 229 (1999) 152–178, http://dx.doi.org/10.1016/S0076-6879(99)99017-1.
[37] L.L. Chaillou, M.A. Nazareno, New method to determine antioxidant activity of polyphenols, J. Agric. Food Chem. 54 (2006) 8397–8402, http://dx.doi.org/10.1021/jf061729f.
[38] E.J. Cáceres, Métodos de preparación de algas para su observación con microscopía electrónica de transmisión convencional (METC), in: K. Alveal, M.E. Ferrario, E.C. Oliveira, E. Sar (Eds.), Manual de Métodos Ficológicos, Universidad de Concepción, Concepción, Chile, 1995, pp. 147–168.
[39] V. Sorrivas de Lozano, A. Morales, Introducción a la microscopía electrónica, Centro Regional de Investigaciones Básicas y Aplicadas de Bahía Blanca Press (CRIBABB), Bahía Blanca, Argentina, 1986, p. 220.
[40] E.C. Oliveira, E.J. Paula, E.M. Plastino, R. Petti, Manual de métodos ficológicos, in: K. Alveal, M.E. Ferrario, E.C. Oliveira, E. Sar (Eds.), Metodologias para cultivo no axénico de macroalgas marinas in vitro, Universidad de Concepción, Concepción, 1995, pp. 429–447.
[41] W.N. Venables, B.D. Ripley, Modern applied statistics with S, Fourth edition, Springer, New York, 2002 (ISBN 0-387-95457-0).
[42] D. Bates, M. Maechler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4, J. Stat. Softw. 67 (2015) 1–48, http://dx.doi.org/10.18637/jss.v067.i01.
[43] S. Jackman, pscl: Classes and Methods for R Developed in the Political Science Computational Laboratory, Department of Political Science, Stanford University, Stanford, California, 2015 (R package version 1.4.9. URL: http://pscl.stanford.edu/).
[44] J. Pinheiro, D. Bates, S. DebRoy, D. Sarkar, R.C. Team, nlme: linear and nonlinear mixed effects models. R package version 3, 2016, pp. 1–128, URL http://CRAN.R-project.org/package=nlme.
[45] J.S. Lefcheck, piecewiseSEM: piecewise structural equation modelling in R for ecology, evolution, and systematics, Methods Ecol. Evol. 7 (2016) 573–579, http://dx.doi.org/10.1111/2041-210X.12512.
[46] T. Hothorn, F. Bretz, P. Westfall, Simultaneous inference in general parametric models, Biom. J. 50 (2008) 346–363, http://dx.doi.org/10.1002/bimj.200810425.
[47] A. Jiménez-Escrig, F.J. Sánchez-Muñiz, Dietary fiber from edible seaweeds: chemical structure, physicochemical properties and effects on cholesterol metabolism, Nutr. Res. 20 (2000) 595–598, http://dx.doi.org/10.1016/S0271-5317(00)00149-4.
[48] H. Funahashi, T. Imai, T. Mase, M. Sekiya, K. Yokoi, H. Hayashi, A. Shibata, T. Hayashi, M. Nishikawa, N. Suda, Y. Hibi, Y. Mizuno, K. Tsukamura, A. Hayakawa, S. Tanuma, Seaweed prevents breast cancer? J. Cancer Res. Ther. 92 (2001) 483–487, http://dx.doi.org/10.1111/j.1349-7006.2001.tb01119.x.
[49] Y.V. Yuan, N.A. Walsh, Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds, Food Chem. Toxicol. 44 (2006) 1144–1150, http://dx.doi.org/10.1016/j.fct.2006.02.002.
[50] B. Larsen, D.M.S.A. Salem, M.A.E. Sallam, M.M. Mishrikey, A.I. Beltagy, Characterization of alginates from algae harvested at the Egyptian Red Sea Coast, Carbohydr. Res. 338 (2003) 2325–2336, http://dx.doi.org/10.1016/S0008-6215(03)00378-1.
[51] I.H. Kim, J.H. Lee, Antimicrobial activities against methicillinresistant *Staphylococcus aureus* from macroalgae, J. Ind. Eng. Chem. 14 (2008) 568–572.
[52] E. Nishide, H. Uchida, Effects of Ulva powder on the ingestion and excretion of cholesterol in rats, in: A.R.O. Chapman, R.J. Anderson, V.J. Vreeland, I.R. Davison (Eds.), Proceedings of the 17th International Seaweed Symposium, Oxford University Press, Oxford, 2003, pp. 165–168.
[53] K. Ito, K. Hori, Seaweed: chemical composition and potential food uses, Food Rev. Int. 5 (1989) 101–144.
[54] J. Fleurence, Seaweed proteins: biochemical, nutritional aspects and potential uses, Trends Food Sci. Technol. 10 (1999) 25–28, http://dx.doi.org/10.1016/S0924-2244(99)00015-1.
[55] J. Fleurence, Seaweed proteins, in: R.Y. Yada (Ed.), Proteins in Food Processing, Woodhead Publishing, Cambridge, 2004, pp. 197–210.
[56] S. Mabeau, J. Fleurence, Seaweed in food products: biochemical and nutritional aspects, Trends Food Sci. Technol. 4 (1993) 103–107.
[57] M. Murata, J. Nakazoe, Production and use of marine algae in Japan, Jpn. Agr. Res. Q. 35 (2001) 281–290, http://dx.doi.org/10.6090/jarq.35.281.
[58] C.S. Lobban, P.J. Harrison, Seaweed Ecology and Physiology, Cambridge University Press, Cambridge, 1994, p. 384.
[59] R.S. Rasmussen, M.T. Morrissey, Marine biotechnology for production of food ingredients, in: S.L. Taylor (Ed.), Advances in Food and Nutrition Research, vol. 52, Elsevier, New York, 2007, pp. 237–292.
[60] S. Chernomorsky, A. Segelman, R.D. Poretz, Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth, Teratog. Carcinog. Mutagen. 19 (1999) 313–322.
[61] N.M. Sachindra, E. Sato, H. Maeda, M. Hosokawa, Y. Niwano, M. Kohno, K. Miyashita, Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites, J. Agric. Food Chem. 55 (2007) 8516–8522 (DOI:10.10077s13197-010-0022-4).
[62] J. Okuzumi, T. Takahashi, T. Yamane, Y. Kitao, M. Inagake, K. Ohya, H. Nishino, Y. Tanaka, Inhibitory effects of fucoxanthin, a natural carotenoid, on N-ethyl-*N'*-nitro-*N*-nitrosoguanidineinduced mouse duodenal carcinogenesis, Cancer Lett. 68 (1993) 159–168.
[63] S. Chinnadurai, G. Kalyanasundaram, Estimation of major pigment content in seaweeds collected from Pondicherry coast, Int. J. Sci. Technol. 9 (2013) 522–525.
[64] L. Bravo, Polyphenols: chemistry, dietary source, metabolism, and nutritional significance, Nutr. Rev. 56 (1998) 317–333.
[65] W.W. Zhang, X.J. Duan, H.L. Huang, Y. Zhang, B.G. Wang, Evaluation of 28 marine algae from the Qingdao coast for antioxidative capacity and determination of antioxidant efficiency and total phenolic content of fractions and subfractions derived from *Symphyocladia latiuscula* (Rhodomelaceae), J. Appl. Phycol. 19 (2007) 97–108, http://dx.doi.org/10.1007/s10811-006-9115-x.
[66] M. Zubia, D. Robledo, Y. Freile-Pelegrin, Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula. Mexico, J. Appl. Phycol. 19 (2007) 449–458, http://dx.doi.org/10.1007/s10811-006-9152-5.
[67] B.P. Kinlan, S.D. Gaines, Propagule dispersal in marine and terrestrial environments: a community perspective, Ecology 84 (2003) 2007–2020, http://dx.doi.org/10.1890/01-0622.
[68] J. Muñoz, Y. Freile-Pelegrín, D. Robledo, Mariculture of *Kappaphycus alvarezii* (Rhodophyta, Solieriaceae) color strains in tropical waters of Yucatán, México, Aquaculture 239 (2004) 161–177, http://dx.doi.org/10.1016/j.aquaculture.2004.05.043.
[69] K. Bogaert, T. Beeckman, O. De Clerck, Abiotic regulation of growth and fertility in the sporophyte of *Dictyota dichotoma* (Hudson) J.V. Lamouroux (Dictyotales, Phaeophyceae), J. Appl. Phycol. 28 (2016) 2915–2924, http://dx.doi.org/10.1371/journal.pone.0061977.
[70] B. Santelices, D. Aedo, A. Hoffman, Banks of microscopic forms and survival to darkness of propagules and microscopic stages of macroalgae, Rev. Chil. Hist. Nat. 75 (2002) 547–555.
[71] L.T. Carney, M.S. Edwards, Role of nutrient fluctuations and delayed development in gametophyte reproduction by *Macrocystis pyrifera* (Phaeophyceae) in southern California, J. Phycol. 46 (2010) 987–996, http://dx.doi.org/10.4490/ALGAE.2006.21.2.161.
[72] K.W. Demes, E. Carrington, J. Gosline, P.T. Martone, Variation in anatomical and material properties explains differences in hydrodynamic performances of foliose red macroalgae (Rhodophyta), J. Phycol. 47 (2011) 1360–1367, http://dx.doi.org/10.1111/j.1529-8817.2011.01066.x.
[73] K.J. McGlathery, R. Marino, R.W. Howarth, Variable rates of phosphate uptake by shallow marine carbonate sediments: mechanisms and ecological significance, Biogeochemistry 25 (1994) 127–146, http://dx.doi.org/10.1007/BF00000882.
[74] C. Oviatt, P. Doering, B. Nowicki, L. Reed, J. Cole, J. Frithsen, An ecosystem level experiment on nutrient limitation in temperate coastal marine environments, Mar. Ecol. Prog. Ser. 116 (1995) 171–179.
[75] R.W. Howarth, D. Anderson, J. Cloern, C. Elfring, C. Hopkinson, B. Lapointe,

T. Malone, N. Marcus, K. McGlathery, A.N. Sharpley, D. Walker, Nutrient pollution of coastal rivers, bays, and seas, Issues Ecol. 7 (2000) 1–15.
[76] B. Lapointe, B. Bedford, Ecology and nutrition of invasive *Caulerpa brachypus f. parvifolia* blooms on coral reefs off southeast Florida, USA, Harmful Algae 9 (2010) 1–12, http://dx.doi.org/10.1016/j.hal.2009.06.001.
[77] C.D. Amsler, M. Neushul, Chemotactic effects of nutrients on spores of the kelps *Macrocytis pyrifera* and *Pterygophora california*, Mar. Biol. 102 (1989) 557–564.
[78] A. Eggert, Seaweed responses to temperature, in: C. Wiencke, K. Bischof (Eds.), Seaweed Biology: Novel Insights into Ecophysiology, Ecology and Utilization, Ecological Studies, Springer, Heidelberg, 2012, pp. 135–156.
[79] A.F. Peters, Culture studies of a sexual life history in *Myriotrichia clavaeformis* (Phaeophyceae, Dictyosiphonales), Br. Phycol. J. 23 (1988) 299–306.
[80] J. Lubchenco, J. Cubit, Heteromorphic life histories of certain marine algae as adaptations to variations in herbivory, Ecology 61 (1980) 676–687.
[81] J.R. Zupan, J.A. West, Photosynthetic responses to light and temperature of the heteromorphic marine alga *Mastocarpus papillatus*, J. Phycol. 26 (1990) 232–239.
[82] E.M. Cunningham, M.D. Guiry, A.M. Breeman, Environmental regulation of development, life history and biogeography of *Helminthora stackhousei* (Rhodophyta) by day length and temperature, J. Exp. Mar. Biol. Ecol. 171 (1993) 1–21, http://dx.doi.org/10.1016/0022-0981(93)90136-C.
[83] L. Couceiro, M. Le Gac, H.M. Hunsperger, S. Mauger, C. Destombe, J.M. Cock, S. Ahmed, S.M. Coelho, M. Valero, A.F. Peters, Evolution and maintenance of haploid-diploid life cycles in natural populations: the case of the marine brown alga *Ectocarpus*, Evolution 69 (2015) 1808–1822.
[84] M.H. Graham, Effect of high irradiance on recruitment of the giant kelp *Macrocystis* (Phaeophyta) in shallow waters, J. Phycol. 32 (1996) 903–906, http://dx.doi.org/10.1111/j.0022-3646.1996.00903.x.
[85] A.J. Hoffmann, M.E. Malbrán, Temperature, photoperiod and light interactions on growth and fertility of *Glossophora kunthii* (Phaeophyta, Dictyotales) from Central Chile, J. Phycol. 25 (1989) 129–133.
[86] I.K. Hwang, H.S. Kim, W.J. Lee, Polymorphism in the brown alga *Dictyota dichotoma* (Dictyotales, Phaeophyceae) from Korea, Mar. Biol. 147 (2005) 999–1015, http://dx.doi.org/10.1007/s00227-005-1623-8.