

2016-04-01

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<http://hdl.handle.net/10026.1/4282>

10.1016/j.marenvres.2015.11.014

MARINE ENVIRONMENTAL RESEARCH

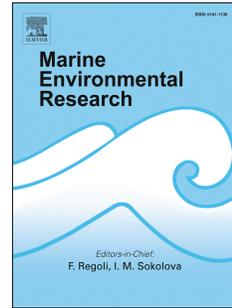
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Accepted Manuscript

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PII: S0141-1136(15)30079-9

DOI: [10.1016/j.marenvres.2015.11.014](https://doi.org/10.1016/j.marenvres.2015.11.014)

Reference: MERE 4101

To appear in: *Marine Environmental Research*

Received Date: 18 August 2015

Revised Date: 19 November 2015

Accepted Date: 28 November 2015

Please cite this article as: Celis-Plá, P.S.M., Bouzon, Z.L., Hall-Spencer, J.M., Schmidt, E.C., Korbee, N., Figueroa, F.L., Seasonal biochemical and photophysiological responses in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta), *Marine Environmental Research* (2015), doi: 10.1016/j.marenvres.2015.11.014.

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1 **Seasonal biochemical and photophysiological responses in the**
2 **intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta).**

3
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15

16 **Abstract**

17 Seasonal changes in the biochemistry and photophysiology of the brown macroalga
18 *Cystoseira tamariscifolia* was analyzed in southern Spain. Total carbon and nitrogen
19 contents, phenolic compounds, antioxidant and photosynthetic activities were
20 seasonally determined over two years. Carbon, nitrogen and photoprotective phenolic
21 contents were higher in winter and spring than in summer and autumn. Antioxidant
22 levels were highest in spring and we found a positive correlation between phenolic
23 content and antioxidant activity (EC_{50}). Photosynthetic capacity (ETR_{max}) and
24 photosynthetic efficiency (α_{ETR}) were also highest in spring, and there was a positive
25 correlation between ETR_{max} and the amount of phenols present. Increased irradiance in
26 spring enhanced algal productivity, antioxidant activity and the production of
27 photoprotective compounds but in summer nutrient depletion due to thermal
28 stratification of coastal waters reduced photosynthetic activity and the photoprotective
29 capacity of *C. tamariscifolia*. Electron microscopy showed that phenols occurred in the
30 cytoplasm of cortical cells inside physodes. Spring would be the best period to harvest
31 *C. tamariscifolia* to extract photoprotectors and antioxidants for potential commercial
32 uses, although the environmental impacts would need to be carefully assessed.

33
34 **Keywords:** Algal productivity, antioxidants, *Cystoseira tamariscifolia*, *in vivo*
35 chlorophyll *a* fluorescence, nitrogen, phenols, UV protection, Mediterranean Sea.

38 **INTRODUCTION**

39 Macroalgae in temperate regions, such as southern Spain, are exposed to wide daily
40 and seasonal changes in photosynthetically active radiation (PAR) and ultraviolet (UV)
41 light. They use photoprotective compounds such as carotenoids or polyphenols to help
42 them cope with high light levels (Stengel et al., 2011). Light harvesting carotenoids are
43 highly efficient O₂ scavengers that play an essential role in protection against excess
44 light and photooxidative stress (Cantrell et al., 2003; Stahl and Sies, 2007). Algae can
45 also prevent UV damage using polyphenols which help dissipate light energy (Goss and
46 Jakob, 2010; Hanelt and Figueroa, 2012) and reduce DNA damage (Gómez and
47 Huovinen, 2010); they can decrease metal toxicity by chelating metal ions (Connan et
48 al., 2004; Stengel et al., 2005) and they are effective chemical defences against a wide
49 range of herbivores (Steinberg and Van Altena, 1992).

50 In stressful conditions, phenolics can be released from algal thalli and react rapidly
51 with proteins and carbohydrates to form UV-absorbing exudates (Koivikko et al., 2005;
52 Celis-Plá et al., 2014a). Phenol content can vary in response to environmental changes
53 in factors such as salinity, nutrients, light, and herbivory (Abdala-Díaz et al., 2006;
54 Celis-Plá et al., 2014b). Brown algal phenols have been investigated for their medical
55 benefits, including anti-inflammatory and hyaluronidase inhibitory activities (Vinay and
56 Kim, 2012). A range of brown algal compounds are used in products as antioxidants
57 Ahn et al., 2007) and for purported benefits as photoprotectors, as antiplasmin
58 inhibitors, to reduce allergies, for skin whitening, anti-HIV-1, antibacterial, and
59 anticancer activities (Sugiura et al., 2007; Artan et al., 2008; Le et al., 2009; Heo et al.,
60 2010).

61 Here, we studied *Cystoseira tamariscifolia* (Hudson) Papenfuss, (Phaeophyceae,
62 Fucales) which can be abundant in waters of high ecological status in the
63 Mediterranean, according to the criteria of Water Framework Directive of the European
64 Union (WFD, 2000/60/EC), and it is used as an indicator of waters with high water
65 quality (Ballesteros et al., 2007, Arévalo et al., 2007, Bermejo et al., 2013). In addition,
66 *C. tamariscifolia* was selected since it provides habitat for other species in the
67 Mediterranean Sea (Bermejo et al., 2013). We used the following well established suite
68 of physiological indicators to seasonally evaluate *C. tamariscifolia* on intertidal rocky
69 shores (according to Figueroa and Korbee, 2010): the C: N stoichiometric ratio as an
70 indicator of nutritional status and phenolic content (Celis-Plá et al., 2014a). Maximum
71 quantum yield of PSII (F_v/F_m) was used to determine photoinhibition and the

72 physiological status of the macroalga (Schreiber et al. 1986). Electron transport rate
73 (ETR) was used to estimate of photosynthetic capacity (Figueroa et al., 2003). In
74 addition, we examined the cell ultrastructure of *C. tamariscifolia* in summer by using
75 both light and transmission electron microcopy to determine the location of phenolic
76 compounds.

77 Our aim was to assess seasonal variability in photosynthetic production and of
78 commercially valuable compounds in *C. tamariscifolia* to inform potential exploitation
79 of these resources. We also investigated seasonal variations in photosynthetic activity,
80 polyphenol content and antioxidant activity.

81

82

83 MATERIALS AND METHODS

84 *Sampling*

85 Nine *Cystoseira tamariscifolia* thalli were collected at least 2 m apart at 0.1-0.4 m
86 above Chart Datum at 10 am local time monthly from July 2012 to June 2014 in
87 summer, autumn, winter and spring. The samples were collected from rocky shores on
88 La Araña beach, Malaga, Spain (36° 45'N, 4° 18'W). Live material was transported in
89 cooled containers and samples were frozen *in situ* using liquid nitrogen for biochemical
90 analyses. Photographs of the habitat were taken in spring, summer, autumn and winter.
91 A representative image for each season of the intertidal area is shown in Figure 1.

92

93 *Abiotic parameters*

94 Photosynthetically active radiation (PAR, $\lambda=400-700$ nm), Ultraviolet A radiation
95 ($\lambda=320-400$ nm) and Ultraviolet B radiation ($\lambda=280-320$ nm) were measured using an
96 UV-PAR Multifilter radiometer NILU-6 (Geminali AS, Oslo, Norway) on the roof of
97 the building of Central Services for Research support (SCAI, University of Malaga)
98 located 14 km from the algal collection site. Seawater temperature was logged every
99 minute at a monitoring station (REDCOS buoy number 1514) located at 36° 42'N, 4°
100 19'W. Seawater nitrate ($\mu\text{mol L}^{-1}$), ammonium ($\mu\text{mol L}^{-1}$), phosphate ($\mu\text{mol L}^{-1}$) and N:
101 P ratio data were obtained from Ramírez et al. (2005) and Mercado et al. (2007, 2012)
102 from 36° 60'N, 4° 10'W.

103

104 *Histology*

105 Macroalgal tissue samples ca 5 mm in length were collected in summer 2013 for
106 Transmission Electron Microscopy, and there were fixed with 2.5% glutaraldehyde,
107 2.0% paraformaldehyde, and 5 mM CaCl₂ in 0.075 M sodium cacodylate buffer (pH
108 7.2) plus 0.2 M sucrose and caffeine 1% overnight. The material was then fixed with
109 1% osmium tetroxide for 4 hours, dehydrated in a graded acetone series and embedded
110 in Spurr's resin. Thin sections were stained with aqueous uranyl acetate followed by
111 lead citrate. Four replicates were made for each experimental group; two samples per
112 replicate were then examined under TEM (JEM 1011 JEOL Ltd., Tokyo, Japan, at 80
113 kV).

114

115 *Biochemical variables*

116 The dry weight of algal carbon and nitrogen contents was determined using an
117 element analyzer (model CNHS-932, LECO Corporation, Michigan, USA). Polyphenol
118 concentrations were measured using 0.25 g fresh weight samples pulverized in a pestle
119 and mortar with sea-sand using 2.5 mL of 80% methanol. This mixture was stored
120 overnight at 4°C then centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was
121 collected to measure the phenolic compound content colourimetrically using Folin-
122 Ciocalteu reagent. Phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) was used as
123 standard. Finally, the absorbance was determined at 760 nm using a Shimadzu UVMini-
124 1240 spectrophotometer (Celis-Plá et al. 2014a). Phenolic concentration was expressed
125 as mg g⁻¹ dry weight after determining the fresh to dry weight ratio in the tissue (the
126 ratio was 5.6). The results are expressed as average ± Standard Error from 9 replicates.

127 The antioxidant activity DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (i.e. EC₅₀)
128 according to Blois (1958) was estimated by reducing the stable free radical DPPH. The
129 supernatant used for phenolic compound measurements was used for DPPH analysis;
130 150 µL of DPPH were added to each extract. This solution of DPPH was prepared in
131 90% methanol (90MeOH: 10H₂O) in 20 mL to concentration 1.27 mM. The reaction
132 was complete after 30 min in a dark room at ~20° C and the absorbance was read at 517
133 nm in a spectrophotometer (UVMini-1240 model, Shimadzu, Columbia, USA). A
134 calibration curve made with DPPH was used to calculate the remaining concentration of
135 DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM)
136 were plotted against plant extract concentration (mg DW mL⁻¹) to obtain the oxidation
137 index EC₅₀, which represents the concentration of the extract, expressed as mg DW

138 mL⁻¹, required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was
 139 used as positive control (Celis-Plá et al., 2014b).

140

141 ***Photosynthesis and energy dissipation as in vivo chlorophyll a fluorescence***

142 *In vivo* chlorophyll *a* fluorescence by Photosystem II was determined using a
 143 portable pulse amplitude modulated fluorometer (Diving-PAM, Walz GmbH,
 144 Germany). Apical pieces of macroalgal thalli were put in 10 mL incubation chambers to
 145 obtain rapid light curves for each treatment. F_o and F_m were measured after 15 minutes
 146 in darkness to obtain the maximum quantum yield (F_v/F_m) being $F_v = F_m - F_o$, F_o the basal
 147 fluorescence of 15 min dark adapted thalli and F_m maximal fluorescence after a
 148 saturation light pulse of $>4000 \mu\text{mol m}^{-2} \text{s}^{-1}$, with a few seconds of the duration
 149 (Schreiber et al., 1995). The electron transport rate (ETR) was determined after 20 s
 150 exposure in twelve increasing irradiances of actinic white light (halogen lamp provided
 151 by the Diving-PAM) (Celis-Plá et al., 2014a). The ETR was calculated as follows
 152 (Schreiber et al., 1995):

153

$$154 \quad \text{ETR} (\mu\text{mol electrons m}^{-2} \text{s}^{-1}) = \Delta F / F'_m \times E \times A \times F_{II} \quad (1)$$

155

156 where $\Delta F / F'_m$ is the effective quantum yield, being $\Delta F = F_m' - F_t$ (F_t is the intrinsic
 157 fluorescence of alga incubated in light and F_m' is the maximal fluorescence reached
 158 after a saturation pulse of algae incubated in light), E is the incident PAR irradiance
 159 expressed in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, A is the thallus absorptance as the fraction of
 160 incident irradiance that is absorbed by the algae (Figueroa et al., 2003) and F_{II} is the
 161 fraction of chlorophyll related to PSII (400-700 nm) being 0.8 in brown macroalgae
 162 (Figueroa et al., 2014a). ETR parameters as maximum electron transport rate (ETR_{max})
 163 and the initial slope of ETR versus irradiance function (α_{ETR}) as estimator of
 164 photosynthetic efficiency were obtained from the tangential function (Eilers and
 165 Peeters, 1988). Finally, the saturation irradiance for ETR ($E_{k_{\text{ETR}}}$) was calculated from
 166 the intercept between ETR_{max} and α_{ETR} . Non-photochemical quenching (NPQ) was
 167 calculated as (Schreiber et al., 1995):

168

$$169 \quad \text{NPQ} = (F_m - F_m') / F_m' \quad (2)$$

170

171 Maximal NPQ (NPQ_{max}) and the initial slope of NPQ *versus* irradiance function
172 (α_{NPQ}) were obtained from the tangential function of NPQ *versus* irradiance (Eilers and
173 Peeters, 1988).

174

175 *Statistical analyses*

176 Pearson correlation coefficients were calculated and tested between all measured
177 dependent variables. Interactive effects between physiological variables were analyzed
178 using ANOVA (according to Underwood, 1997). This test was performed for *C.*
179 *tamariscifolia* including year and season (two-way) as fixed factors for biochemical
180 variables (mean \pm SE, $n=9$) and season (one-way) with four levels, for the
181 photosynthetic variables (mean \pm SE, $n=9$), with a level of probability applied in the
182 statistical analyses at $P<0.05$. Homogeneity of variance was tested using Cochran tests
183 and by visual inspection of the residuals. Student Newman-Keuls tests (SNK) were
184 performed after significant ANOVA interactions. All data conformed to homogeneity of
185 variance. Analyses were carried out using SPSS v.21 (IBM, USA). The general
186 variation patterns between biochemical variables measured in *C. tamariscifolia* were
187 explored using a multivariate approach. A Principal Coordinates Analysis (PCO) was
188 performed for this purpose on the basis of Euclidean distance using PERMANOVA+
189 for PRIMER6 package (Anderson et al. 2008). Such multivariate ordination was used
190 because it allowed for investigating the variation of the content of biochemical
191 compounds at the same time by looking at the ordination plot. Each one of variables
192 was represented by an arrow in the ordination plot pointing to the samples that showed
193 the highest amount of that particular compound. Each replicate represented the content
194 of all compounds calculated from the three thalli taken at one sampling for each month
195 and grouped for season.

196

197 **RESULTS**

198 *Environmental conditions*

199 *Cystoseira tamariscifolia* was abundant in all seasons whereas *Ulva rigida*
200 (Chlorophyta) was only abundant in the summer. More *C. tamariscifolia* was present in
201 spring in respect to other species such as e.g., *Ellisolandia elongata* (Figure 1). The
202 seawater temperature ranged from 14-23°C (Table 1) with a peak summer average daily
203 irradiance of *ca.* 10165 kJ m⁻² for PAR, 1051 kJ m⁻² for UVA and 57.5 kJ m⁻² UVB
204 (Figure 2A-C). Seasonal nitrate (NO_3^-) concentrations ranged from 0.6-1.5 mg L⁻¹ in

205 this coastal area according to Ramírez et al. (2005) and Mercado et al. (2007 and 2012).
206 Seawater nitrate concentrations are approximately 2.5 times higher in winter and spring
207 than in summer and autumn (Table 1). Ammonium (NH_4^+) varied through the year from
208 0.1 to 0.5 mg L^{-1} and was 2.7 times higher in summer than in autumn and winter, 1.4
209 times higher than in spring (Table 1). The phosphate (PO_4^{3-}) concentration varied little
210 (0.12 to 0.15 mg L^{-1}) in all seasons (Table 1). Chlorophyll *a* concentrations were highest
211 in spring with 1.45 mg L^{-1} and lowest in summer with 0.92 mg L^{-1} , respectively (Table
212 1).

213

214 ***Morphological observations***

215 The cortical cells of *C. tamariscifolia* had numerous chloroplasts (Figure 3A) and
216 physodes with a thick cell wall (Figures 3A and C) that was embedded with phenolic
217 compounds (Figure 3B). Mitochondria were associated with the chloroplasts (Figure
218 3D) which had the typical internal organization of brown algae with thylakoids
219 aggregated in bands (Figure 3D). Lipid droplets (plastoglobuli) were situated between
220 the thylakoids (Figure 3D) and there were plasmodesmata cell connections (Figure 3E).

221

222 ***Biochemical responses***

223 Principal Coordinates Analysis (PCO) (Figure 4) revealed a positive correlation of
224 the first axis (43.8% of total variation) with the internal N content. In contrast, the ratio
225 C:N, antioxidant activity and phenolic compounds were negatively correlated with this
226 axis. Seasonality had a marked effect upon these factors (Figure 4). Moreover, the
227 combination of the first two axes explained the 79.5% of the variation in these variables
228 (Figure 4). The small angles between the arrows are indicative of high correlation
229 between the variables. The carbon and nitrogen contents of *C. tamariscifolia* were
230 significantly higher in winter and spring and the C: N ratio was significantly lower in
231 winter (Figure 5, Table 3). Phenol content and antioxidant activity (i.e. less EC_{50}) were
232 significantly higher in spring (Figure 6, Table 3).

233

234 ***Physiological responses***

235 The F_v/F_m ratio was not significantly affected by season, although it tended to be
236 higher in winter. Maximal electron transport rate (ETR_{max}) was highest in spring and
237 photosynthetic efficiency (α_{ETR}) was significantly lower in winter (Tables 2 and 4). The
238 irradiance of saturation of curve (Ek_{ETR}) was not significantly affected by season, but

239 tended to be higher in winter and spring. The highest non-photochemical quenching
240 (NPQ_{max}) occurred winter, although no statistically significant seasonal differences were
241 found (Tables 2 and 4). The irradiance of saturation of non-photochemical quenching
242 (E_{kNPQ}) was significantly higher in autumn and the ratio ETR_{max} (production): NPQ_{max}
243 (photoprotection) was highest in spring (Tables 2 and 4). Positive correlations between
244 phenolic compounds and antioxidant activity and between antioxidant activity and
245 nitrogen internal content, through all seasons, were found. There was also a positive
246 correlation between EC_{50} , ETR_{max} and photosynthetic efficiency (Table S1). The
247 absorbance tended to be higher in winter and spring. ETR_{max} and phenolic content was
248 also positively correlated.

249

250 DISCUSSION

251 We found that as the short days of winter lengthened into spring this stimulated an
252 upsurge in photoprotectors, antioxidants, and productivity in *Cystoseira tamariscifolia*
253 as the algae laid down stores of nitrogen and carbon. The phenol and antioxidant
254 capacity of this seaweed fell in summer which we attribute to nutrient depletion as the
255 sea surface waters became gradually more oligotrophic due to thermal stratification. Our
256 analyses of nitrogen and carbon contents revealed nutrient limitation in summer and
257 autumn. This seaweed accumulated nitrogen during winter and spring as a reservoir for
258 periods of the high irradiance when photoprotective mechanisms are most needed
259 (Figueroa et al., 2014b; Celis-Plá et al., 2014a). Increased photosynthetic activity can
260 enhance the accumulation of phenolic compounds in *C. tamariscifolia* as reported in
261 other brown algae (Pavia and Toth 2000) as well as in the green alga *Ulva rigida*
262 (Cabello-Pasini et al. 2011).

263 We attribute declining phenolic content in *C. tamariscifolia* in the summer to light
264 damage when both PAR and UV radiation peaks (Stengel et al., 2014). Phenol release
265 increases at noon in summer daily cycles (Abdala-Díaz et al., 2006). High PAR
266 irradiances and emersion have been associated with increasing phlorotannin release
267 rates (Ragan and Jensen 1978; Carlson and Carlson 1984). Celis-Plá et al., (2014a) also
268 found a higher release rate of polyphenols from *C. tamariscifolia* in outdoor
269 experiments in summer compared to winter. Phenolic compounds released from the
270 thalli into the seawater can react rapidly with both proteinaceous and carbohydrate
271 substances to form UV-absorbing complexes (Swanson and Druehl 2002; Koivikko et
272 al., 2005). Release of phenolic compounds is thought to be a photoprotection

273 mechanism due to the transient reduction of UV penetration favored by the
274 accumulation of excreted phenols in the cell wall. Defense against epiphytic algae and
275 bacteria (Koivikko et al., 2005). Karban and Baldwin (1997) is thought to be an indirect
276 effect of excreted phlorotannins in algae which are released into the water when algae
277 are grazed.

278 The positive correlation between phenolic compounds and C content with maximal
279 ETR indicates a coupling between photosynthesis and carbon accumulation with
280 secondary metabolism (the accumulation of inducible UV photoprotective compounds
281 as polyphenols under stress conditions). In spring, photosynthetic energy can be used
282 for both accumulation of carbon compounds to store energetic and to build up
283 photoprotective compounds. In contrast, antioxidant carotenoids and polyunsaturated
284 fatty acids accumulate in stressful conditions and decreased photosynthetic activity
285 (Stengel et al., 2011; Sharma et al., 2012). Here we found that phenolic compounds
286 were directly related to maximal photosynthetic productivity (ETR_{max}). The
287 accumulation of phenols in *C. tamariscifolia* under increased PAR and UV irradiances
288 has previously been reported (Abdala-Díaz et al., 2006; Figueroa et al., 2014a). *C.*
289 *tamariscifolia* acclimates to high UVB by up-regulating UV screen substances that also
290 act as antioxidants (Figueroa et al., 2014a). Connan et al., (2004) showed that mid-shore
291 brown algae (such as *Fucus spiralis*, *F. vesiculosus*, *Ascophyllum nodosum*) tend to
292 have higher phenol content and antioxidant activity than those found in the low
293 intertidal or sublittoral zone (such as *F. serratus*, *Bifurcaria bifurcata*, *Himathalia*
294 *elongata* and *Laminaria digitata*) and suggest that this is to protect them against the
295 higher UV irradiance levels of the mid shore.

296 Where nutrient levels permit, brown algal phenols are stimulated by high light
297 levels (Pavia and Brock, 2000) but peak phenol content is often not found in summer
298 since nitrate concentrations can become limiting (Pavia and Åberg, 1996). This
299 certainly seems true for *C. tamariscifolia* which has higher phenolic contents when
300 nitrates are most abundant (Celis-Plá et al., 2014b). We found that *C. tamariscifolia*
301 phenol content peaked in spring at about 5-7.0% which is within the range of the
302 highest levels found in brown algae from northwest Europe (Pavia and Åberg, 1996;
303 Connan et al., 2004) and in *Cystoseira* spp. from other areas of Mediterranean sea
304 (Abdala-Díaz et al., 2006; Celis-Plá et al., 2014b and 2015). Phenol-rich vesicles,
305 known as physodes, were mainly located in cortical cells, as is the case in other
306 seaweeds (Schoenwaelder, 2008; Gómez and Huovinen, 2010). This location provides

307 photoprotection of cytoplasmic organelles and nuclei from both cortical and medullar
308 cells (Schoenwaelder, 2008). In *C. tamariscifolia*, phenols are accumulated
309 preferentially at the apices (Abdala-Díaz et al., 2014), i.e., the part of the thalli with
310 highest light exposure. In our study, in order to avoid the heterogeneity of the phenolic
311 content in the thalli, samples were always collected from the apical part. Phenolic
312 compounds found in *C. tamariscifolia* can be related to photoprotective mechanisms,
313 decreasing the negative effect of increased UVB radiation (Figuroa et al., 2014a).
314 Higher phenol levels occur in brown algae from Southern Chile despite local high light
315 and nutrient levels (Gómez and Huovinen, 2010). These differences can be attributed to
316 higher irradiance in south Spain compared to that of Southern Chile.

317 *Cystoseira tamariscifolia* collected in summer, spring and winter had higher NPQ
318 values than those collected in autumn indicating active photoprotective mechanisms
319 related to the xanthophyll cycle (Demmig-Adams and Adams, 2006). Maximal
320 photosynthetic capacity (i.e. ETR_{max}) and photosynthetic efficiency (i.e. α_{ETR}) were
321 highest in spring when high daily PAR (ca. 102.72 MJm^{-2}) favored more photosynthetic
322 activity than in winter and autumn (ca. 51.5 MJm^{-2}). This indicates that the productivity
323 of *C. tamariscifolia* peaks in spring.

324 Antioxidant, photoprotective, antiplasmin, antiallergic, antiviral antibacterial and
325 anticancer properties have all been reported in brown algae (Sugiura et al., 2007; Artan
326 et al., 2008; Heo et al., 2010). Several seaweed chemicals, such as phloroglucinols from
327 *Ecklonia cava*, are widely used in Asian medicines, foods and cosmetics (Le et al.,
328 2009). The exploitation of these natural resources requires an evaluation of the biomass
329 and content of the algal compounds through the time (Stengel et al., 2011). The seasonal
330 variations in the biochemical composition and physiology of *C. tamariscifolia* can
331 inform management in this species. Spring would be the best period to harvest *C.*
332 *tamariscifolia* to extract photoprotectors and antioxidants for commercial products,
333 although the environmental impacts would need to be carefully assessed. Poorly
334 managed harvesting of macroalgae can seriously impact natural populations (Stagnol et
335 al., 2013) so any exploitation would require impact assessments prior to licensing
336 managed activities.

337

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339

340

341 CONCLUSIONS

342 In *C. tamariscifolia* photosynthetic production peaked in spring when light levels
343 temperature and nutrients were optimal for building up stores of phenols and
344 antioxidants. In summer photoinhibition and low nutrients stressed the *C.*
345 *tamariscifolia*.

346 Any harvesting of this seaweed as a source of phenolic with antioxidant capacity
347 would be best carried out in spring when these chemicals peak in abundance. Further
348 work would be needed to establish protocols for the harvesting of *C. tamariscifolia* to
349 avoid adverse environmental impacts.

350

351 ACKNOWLEDGMENTS

352 This work was supported by the Junta de Andalucía (Project RNM-5750) and by the
353 research group RNM-295. Paula S. M. Celis-Plá gratefully acknowledges financial
354 support from “Becas-Chile” (CONICYT) fellowship of doctorate of the Ministry of
355 Education of the Republic of Chile.

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543 Table 1. Seasonal changes in surface seawater temperature (mean \pm SE, n=2144)
 544 according to REDCOS buoy (number 1514) and salinity, nitrate, ammonium, phosphate
 545 and N: P ratio (mean values \pm SE, n=180) in Málaga bay (Southern Spain) according to
 546 Ramirez et al. (2005) and Mercado et al. (2007, 2012).

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	Units	Summer	Autumn	Winter	Spring
Temperature	°C	18.91 \pm 2.09	17.81 \pm 1.66	14.88 \pm 0.45	15.91 \pm 1.14
Salinity		36.87 \pm 0.29	36.72 \pm 0.34	36.93 \pm 0.28	37.14 \pm 0.45
Nitrate	$\mu\text{mol L}^{-1}$	0.58 \pm 1.07	0.62 \pm 0.77	1.52 \pm 1.07	1.59 \pm 1.44
Ammonium	$\mu\text{mol L}^{-1}$	0.53 \pm 0.75	0.19 \pm 0.27	0.18 \pm 0.10	0.35 \pm 0.20
Phosphate	$\mu\text{mol L}^{-1}$	0.12 \pm 0.08	0.14 \pm 0.01	0.14 \pm 0.05	0.15 \pm 0.09
N:P molar ratio		4.3 \pm 6.6	7.4 \pm 10.9	13.4 \pm 12.3	16.0 \pm 21.3
Chlorophyll <i>a</i>	$\mu\text{mol L}^{-1}$	0.92 \pm 0.69	1.21 \pm 0.94	1.22 \pm 1.14	1.45 \pm 0.99

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564 Table 2. Photosynthetic physiology of *Cystoseira tamariscifolia* collected in La Araña
 565 beach near Málaga (Southern Spain) in summer, autumn, winter and spring 2013-2014.
 566 Maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max} , expressed in
 567 $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$), photosynthetic efficiency (α_{ETR}), irradiance of saturation of
 568 ETR (Ek_{ETR}) expressed in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, maximal non-photochemical quenching
 569 (NPQ_{max}), irradiance of saturation of NPQ (Ek_{NPQ}) expressed in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$
 570 and ETR_{max}/NPQ_{max} ratio (mean \pm SE, n=9). Lower-case letters denote significant
 571 differences after SNK test.
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	<i>Cystoseira tamariscifolia</i>			
	Summer	Autumn	Winter	Spring
F_v/F_m	0.71 \pm 0.01	0.71 \pm 0.02	0.69 \pm 0.02	0.71 \pm 0.01
ETR_{max}	52.18 \pm 3.39 ^a	53.01 \pm 2.23 ^a	55.14 \pm 4.29 ^a	70.65 \pm 6.58 ^b
α_{ETR}	0.41 \pm 0.02 ^b	0.39 \pm 0.01 ^b	0.27 \pm 0.01 ^a	0.36 \pm 0.03 ^b
Ek_{ETR}	137.95 \pm 0.06	136.52 \pm 17.81	235.64 \pm 49.84	272.38 \pm 84.51
NPQ_{max}	1.39 \pm 0.11	1.27 \pm 0.16	1.61 \pm 0.18	1.38 \pm 0.14
Ek_{NPQ}	301.28 \pm 33.22 ^{ab}	395.56 \pm 48.98 ^b	190.99 \pm 27.83 ^a	295.61 \pm 66.12 ^{ab}
ETR_{max}/NPQ_{max}	42.32 \pm 4.91	52.14 \pm 15.68	38.77 \pm 3.93	61.96 \pm 7.43
<i>Absorptance</i>	0.76 \pm 0.04	0.77 \pm 0.02	0.83 \pm 0.02	0.79 \pm 0.02

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584 Table 3. Seasonal and annual effects on the carbon, nitrogen, C:N ratio, phenolic
 585 compounds and antioxidant activity (EC_{50}) of *Cystoseira tamariscifolia* collected in
 586 2012-2014 on a rocky shore near Málaga, southern Spain. Significant differences at $P <$
 587 0.05 are shown in bold.

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		<i>Cystoseira tamariscifolia</i>			
		Df	MS	F	P
<i>Carbon</i>	<i>Year</i>	1	81.83	0.40	0.53
	<i>Season</i>	3	928.75	4.52	<0.01
	<i>Year*Season</i>	3	201.34	0.98	0.41
	<i>Res</i>	64	205.41		
<i>Nitrogen</i>	<i>Year</i>	1	15.11	1.59	0.21
	<i>Season</i>	3	163.24	17.14	<0.01
	<i>Year*Season</i>	3	12.49	1.31	0.28
	<i>Res</i>	64	9.52		
<i>Ratio C:N</i>	<i>Year</i>	1	9.83	1.54	0.22
	<i>Season</i>	3	81.43	12.76	<0.01
	<i>Year*Season</i>	3	11.12	1.74	0.17
	<i>Res</i>	64	6.38		
<i>Phenolic compounds</i>	<i>Year</i>	1	188.25	2.04	0.16
	<i>Season</i>	3	1763.93	19.11	<0.01
	<i>Year*Season</i>	3	1576.40	17.07	<0.01
	<i>Res</i>	64	92.33		
EC_{50}	<i>Year</i>	1	0.00	0.47	0.49
	<i>Season</i>	3	0.04	4.31	<0.01
	<i>Year*Season</i>	3	0.02	1.86	0.15
	<i>Res</i>	64	0.01		

589 *Res: residual*

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598 Table 4. Seasonal effects on *Cystoseira tamariscifolia* photosynthesis on a rocky shore
 599 near Málaga in 2013-2014; maximal quantum yield (F_v/F_m), maximal electron transport
 600 rate (ETR_{max}), photosynthetic efficiency (α_{ETR}), irradiance of saturation of ETR
 601 (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}), irradiance of saturation of
 602 NPQ (Ek_{NPQ}) and relationship between ETR_{max}/NPQ_{max} . Significant differences at $P <$
 603 0.05 are shown in bold.

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		<i>Cystoseira tamariscifolia</i>			
		df	MS	F	P
F_v/F_m	Season	3	0.00	0.19	0.90
	Res	32	0.00		
ETR_{max}	Season	3	680.4	3.8618	0.02
	Res	32	176.2		
α_{ETR}	Season	3	0.03	5.61	<0.01
	Res	32	0.01		
Ek_{ETR}	Season	3	42939.9	1.91	0.15
	Res	32	22502.1		
NPQ_{max}	Season	3	0.18	0.82	0.49
	Res	32	0.21		
Ek_{NPQ}	Season	3	62901.2	3.23	0.04
	Res	32	19464.4		
ETR_{max}/NPQ_{max}	Season	3	691.3	0.90	0.45
	Res	32	767.9		
Absorptance	Season	3	0.01	1.95	0.14
	Res	32	0.00		

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Res: residual

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616 **Figure captions**

617 Figure 1. La Araña rocky shore in southern Spain showing high perennial coverage of
618 the brown alga *Cystoseira tamariscifolia* and spring/summer blooms of *Ulva* spp. in
619 2013.

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621 Figure 2. Daily integrated irradiance per month in the period 2012-2014 of A) PAR
622 (400-700 nm), B) UVA (320-400 nm) and C) UVB (280-320 nm) in the NILU UV6
623 station located in the roof Central Services for Research building (University of
624 Malaga).

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626 Figure 3. Transmission electron microscopy images of *Cystoseira tamariscifolia* from
627 La Araña in summer 2013. A) Cortical cell with many chloroplasts (C), physodes (Ph)
628 and thick cell wall (CW). B) Arrows indicate presence of phenolic compounds in cell
629 wall. C) Detail of physodes in cortical cell. D) Chloroplast with plastoglobuli (P) and
630 associated mitochondria (M). E) Detail of plasmodesmata (arrows).

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632 Figure 4. PCO diagram in relation to Season (spring, summer, autumn and winter).
633 Vectors overlay (Spearman rank correlation) indicates the relationship between the PCO
634 axes and the ecophysiological variables; C, N: internal content and C:N relationship,
635 respectively, PC: phenolic compounds and AA: such as $1/EC_{50}$ antioxidant activity, in
636 the time.

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638 Figure 5. A) Carbon and B) Nitrogen contents expressed as $mg\ g^{-1}$ DW and C) C:N
639 ratio of *Cystoseira tamariscifolia* in summer, autumn, winter and spring.

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641 Figure 6. A) Phenolic compounds (expressed as $mg\ g^{-1}$ DW) and B) Antioxidant
642 activity (EC_{50} ; expressed as $mg\ DW\ mL^{-1}$) to *Cystoseira tamariscifolia* through the
643 season (summer, autumn, winter and spring).

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Summer



Autumn

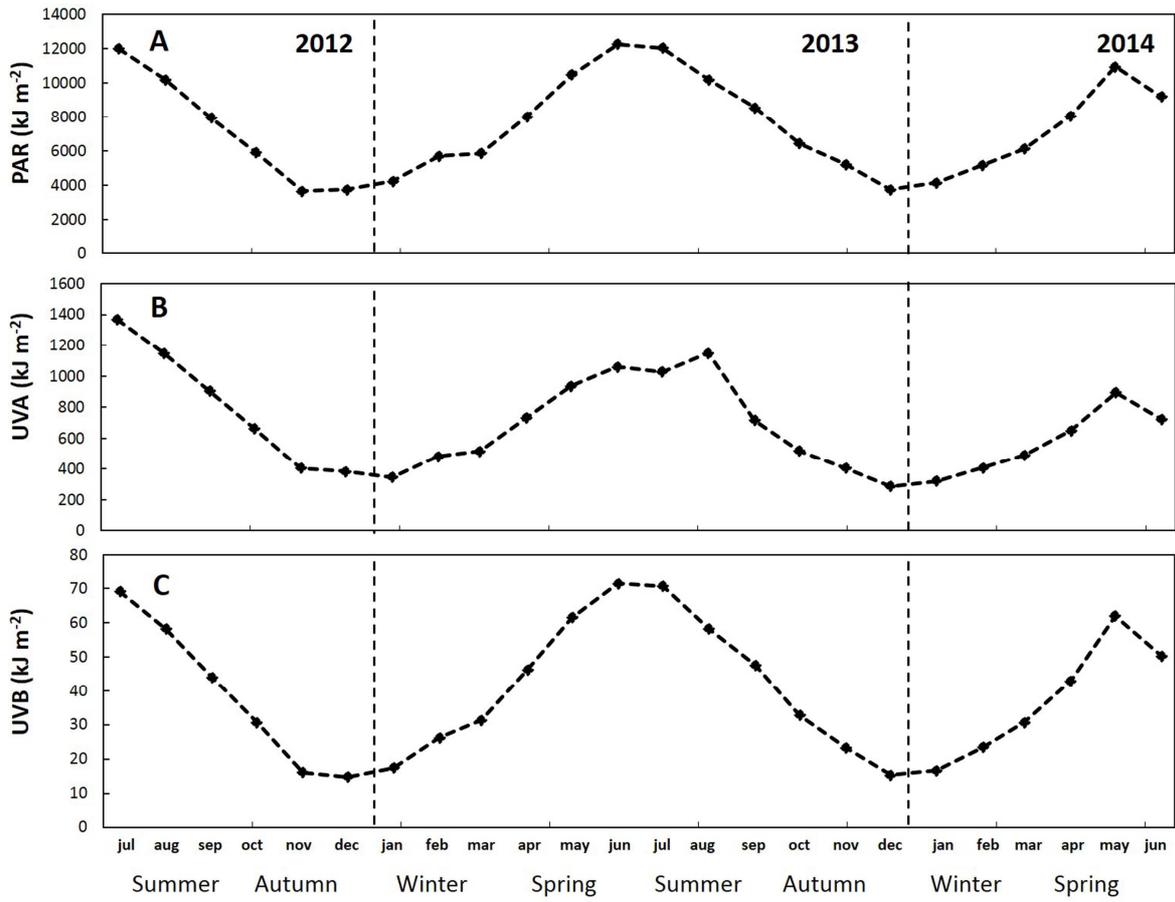


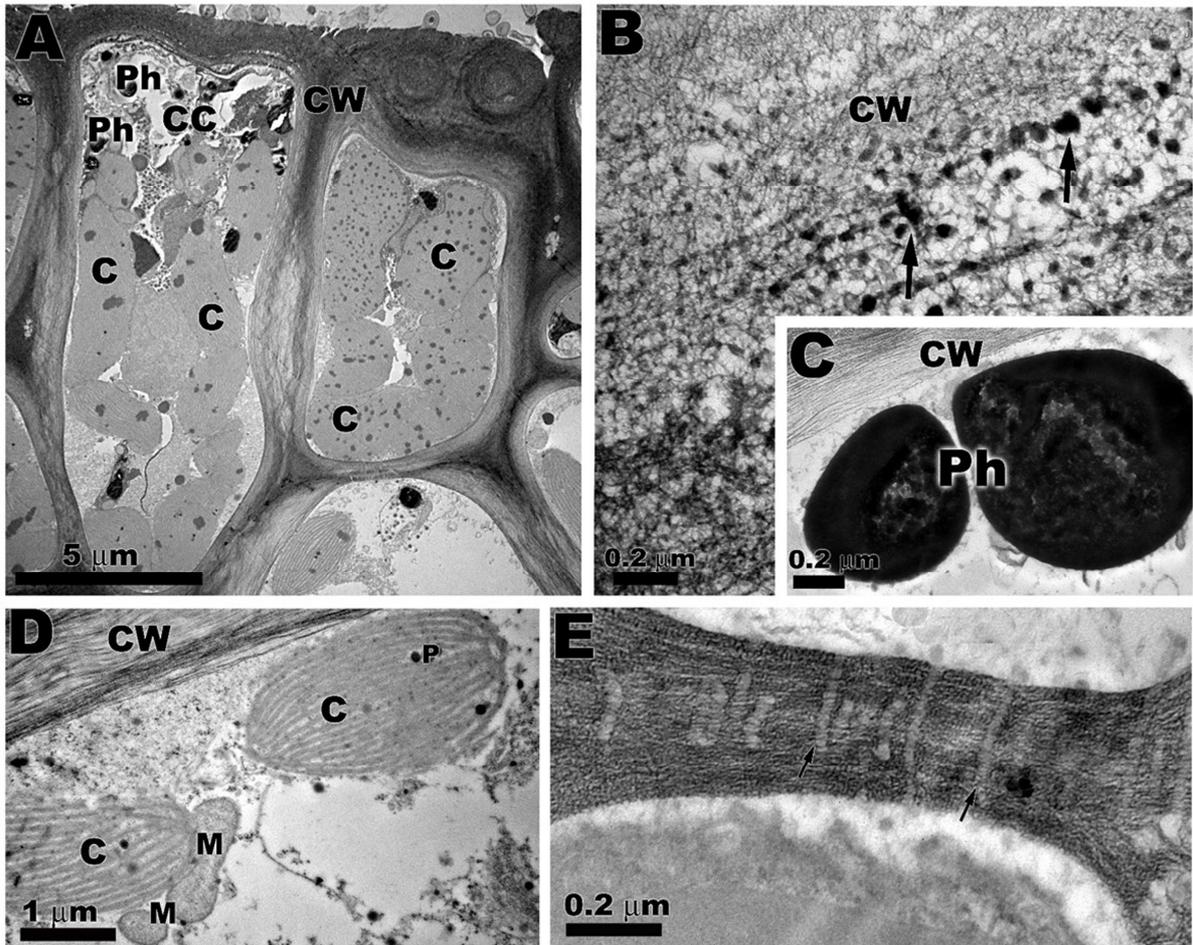
Winter

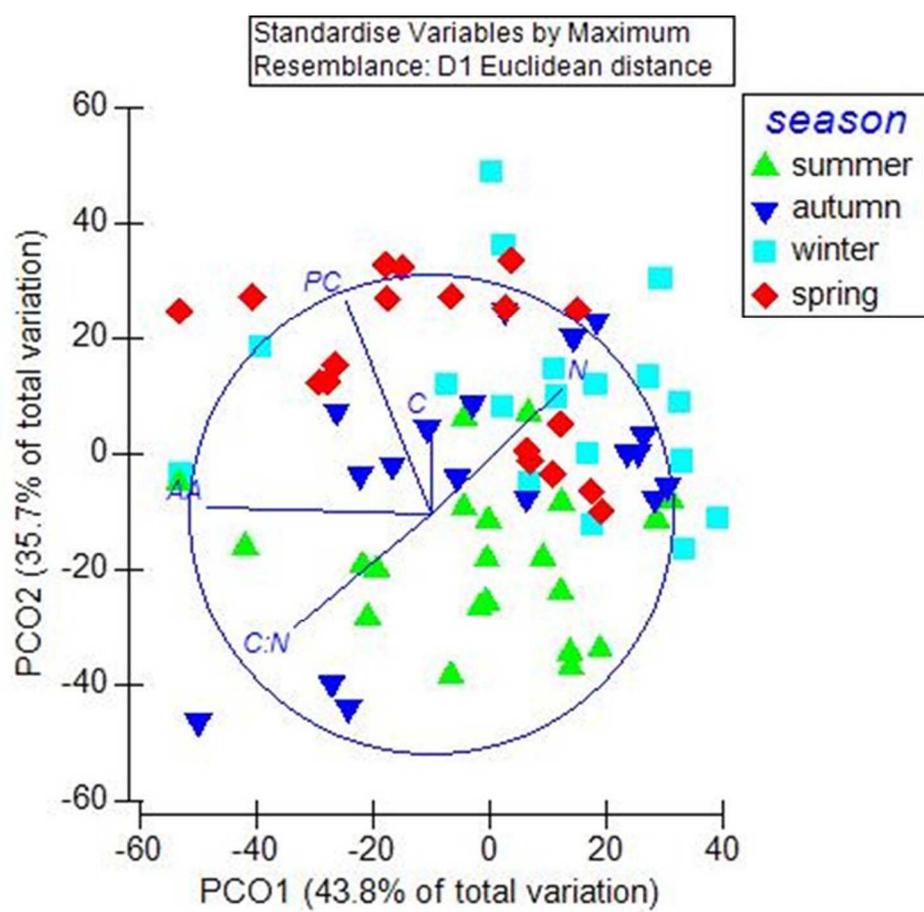


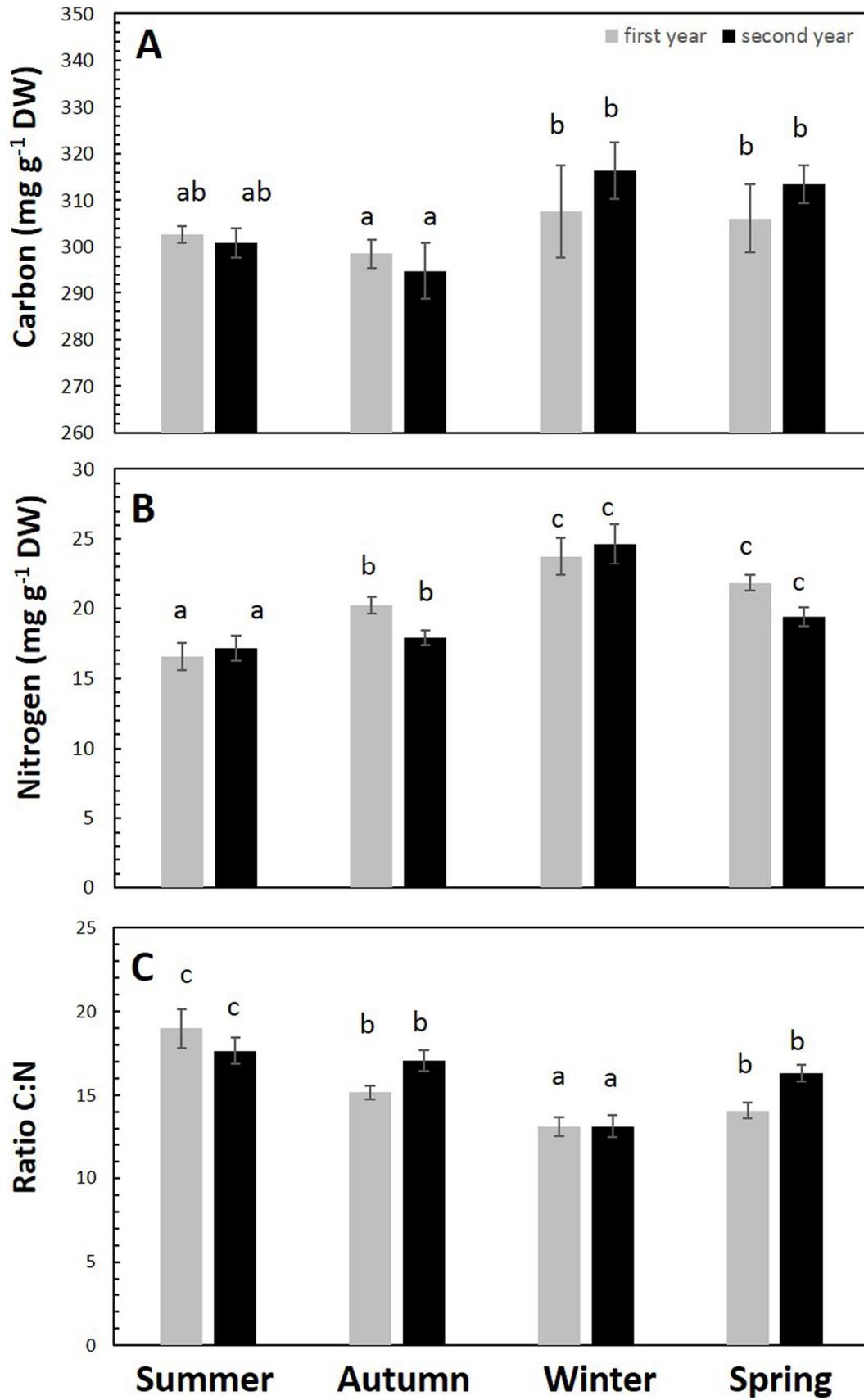
Spring

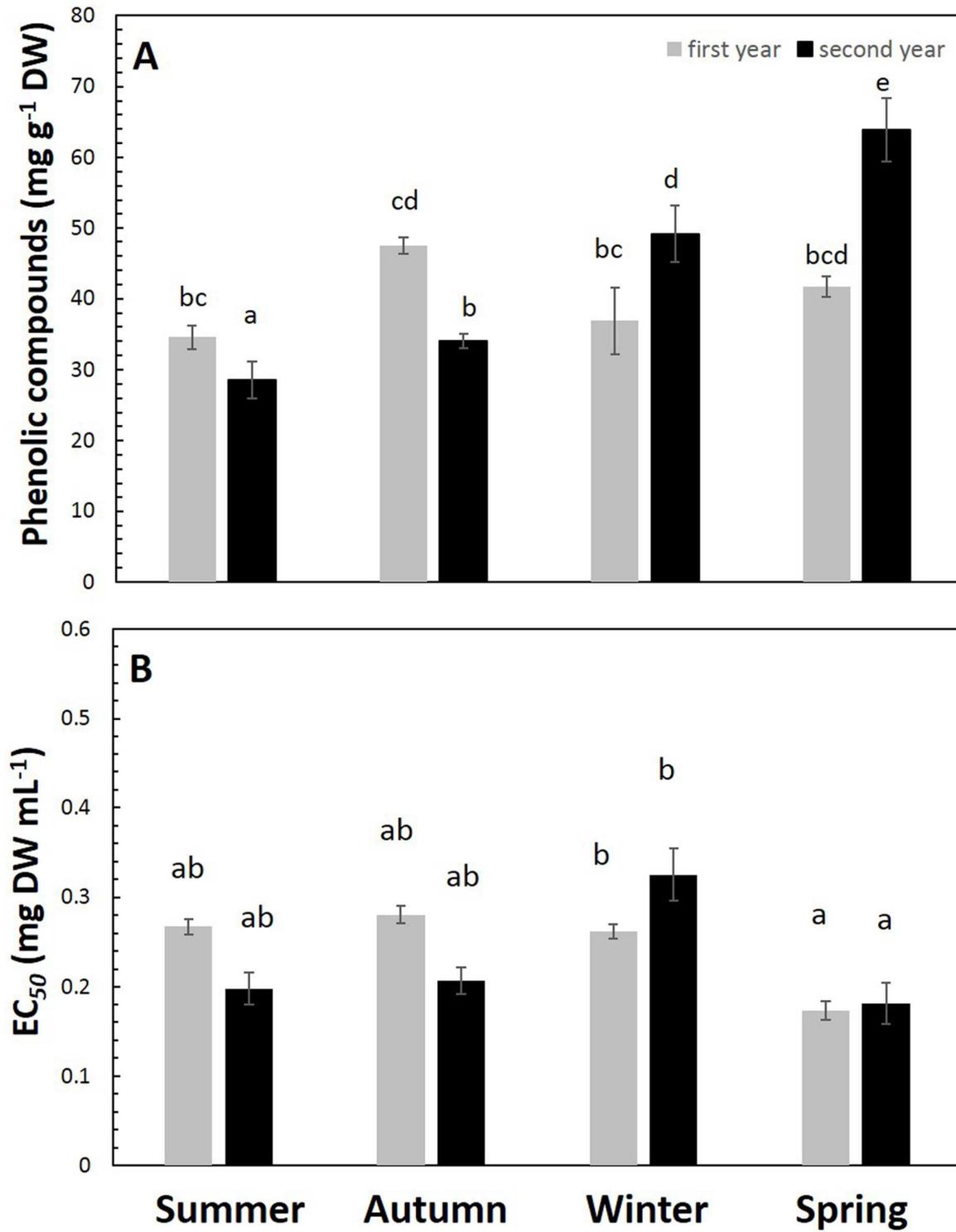












Seasonal biochemical and photophysiological responses in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta).

Paula S.M. Celis-Plá, Zenilda L. Bouzon, Jason M. Hall-Spencer, Eder C. Schmidt,
Nathalie Korbee and Félix L. Figueroa.

Highlights

- Monitoring of the seasonal changes in the biochemistry and photophysiology of the brown macroalga *Cystoseira tamariscifolia*.
- The increased irradiance in spring enhanced the algal productivity, antioxidant activity and the production of photoprotective compounds.
- The monitoring for the best period to harvest *Cystoseira tamariscifolia* to extract potential commercial uses could be in spring.