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# PHOTOPHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF CO<sub>2</sub> AND TEMPERATURE LEVELS ON CYSTOSEIRA TAMARISCIFOLIA, COLLECTED ON SOUTHERN SPAIN

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

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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 ( $\alpha_{ETR}$ ) 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 O<sub>2</sub> 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 CaCl<sub>2</sub> 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<sup>-1</sup> 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. EC<sub>50</sub>)  
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: 10H<sub>2</sub>O) 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<sup>-1</sup>) to obtain the oxidation  
137 index EC<sub>50</sub>, which represents the concentration of the extract, expressed as mg DW

138 mL<sup>-1</sup>, 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 ( $\text{ETR}_{\text{max}}$ )  
 163 and the initial slope of ETR versus irradiance function ( $\alpha_{\text{ETR}}$ ) 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  $\text{ETR}_{\text{max}}$  and  $\alpha_{\text{ETR}}$ . 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 ( $\text{NPQ}_{\text{max}}$ ) and the initial slope of NPQ *versus* irradiance function  
172 ( $\alpha_{\text{NPQ}}$ ) 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<sup>-2</sup> for PAR, 1051 kJ m<sup>-2</sup> for UVA and 57.5 kJ m<sup>-2</sup> UVB  
204 (Figure 2A-C). Seasonal nitrate (NO<sub>3</sub><sup>-</sup>) concentrations ranged from 0.6-1.5 mg L<sup>-1</sup> 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 ( $\text{NH}_4^+$ ) varied through the year from  
208 0.1 to 0.5  $\text{mg L}^{-1}$  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 ( $\text{PO}_4^{3-}$ ) concentration varied little  
210 (0.12 to 0.15  $\text{mg L}^{-1}$ ) in all seasons (Table 1). Chlorophyll *a* concentrations were highest  
211 in spring with 1.45  $\text{mg L}^{-1}$  and lowest in summer with 0.92  $\text{mg L}^{-1}$ , 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  $\text{EC}_{50}$ ) 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 ( $\text{ETR}_{\text{max}}$ ) was highest in spring and  
237 photosynthetic efficiency ( $\alpha_{\text{ETR}}$ ) was significantly lower in winter (Tables 2 and 4). The  
238 irradiance of saturation of curve ( $\text{Ek}_{\text{ETR}}$ ) 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 form 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 form 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.  $\alpha_{ETR}$ ) were  
321 highest in spring when high daily PAR (ca.  $102.72 \text{ MJm}^{-2}$ ) favored more photosynthetic  
322 activity than in winter and autumn (ca.  $51.5 \text{ MJm}^{-2}$ ). 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.

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**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.

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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 ( $\alpha_{ETR}$ ), 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.  
 572

	<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 <sup>a</sup>	53.01 $\pm$ 2.23 <sup>a</sup>	55.14 $\pm$ 4.29 <sup>a</sup>	70.65 $\pm$ 6.58 <sup>b</sup>
$\alpha_{ETR}$	0.41 $\pm$ 0.02 <sup>b</sup>	0.39 $\pm$ 0.01 <sup>b</sup>	0.27 $\pm$ 0.01 <sup>a</sup>	0.36 $\pm$ 0.03 <sup>b</sup>
$Ek_{ETR}$	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 <sup>ab</sup>	395.56 $\pm$ 48.98 <sup>b</sup>	190.99 $\pm$ 27.83 <sup>a</sup>	295.61 $\pm$ 66.12 <sup>ab</sup>
$ETR_{max}/NPQ_{max}$	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	<b>928.75</b>	<b>4.52</b>	<b>&lt;0.01</b>
	<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	<b>163.24</b>	<b>17.14</b>	<b>&lt;0.01</b>
	<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	<b>81.43</b>	<b>12.76</b>	<b>&lt;0.01</b>
	<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	<b>1763.93</b>	<b>19.11</b>	<b>&lt;0.01</b>
	<i>Year*Season</i>	3	<b>1576.40</b>	<b>17.07</b>	<b>&lt;0.01</b>
	<i>Res</i>	64	92.33		
$EC_{50}$	<i>Year</i>	1	0.00	0.47	0.49
	<i>Season</i>	3	<b>0.04</b>	<b>4.31</b>	<b>&lt;0.01</b>
	<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 ( $\alpha_{ETR}$ ), 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	<b>680.4</b>	<b>3.8618</b>	<b>0.02</b>
	Res	32	176.2		
$\alpha_{ETR}$	Season	3	<b>0.03</b>	<b>5.61</b>	<b>&lt;0.01</b>
	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	<b>62901.2</b>	<b>3.23</b>	<b>0.04</b>
	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**



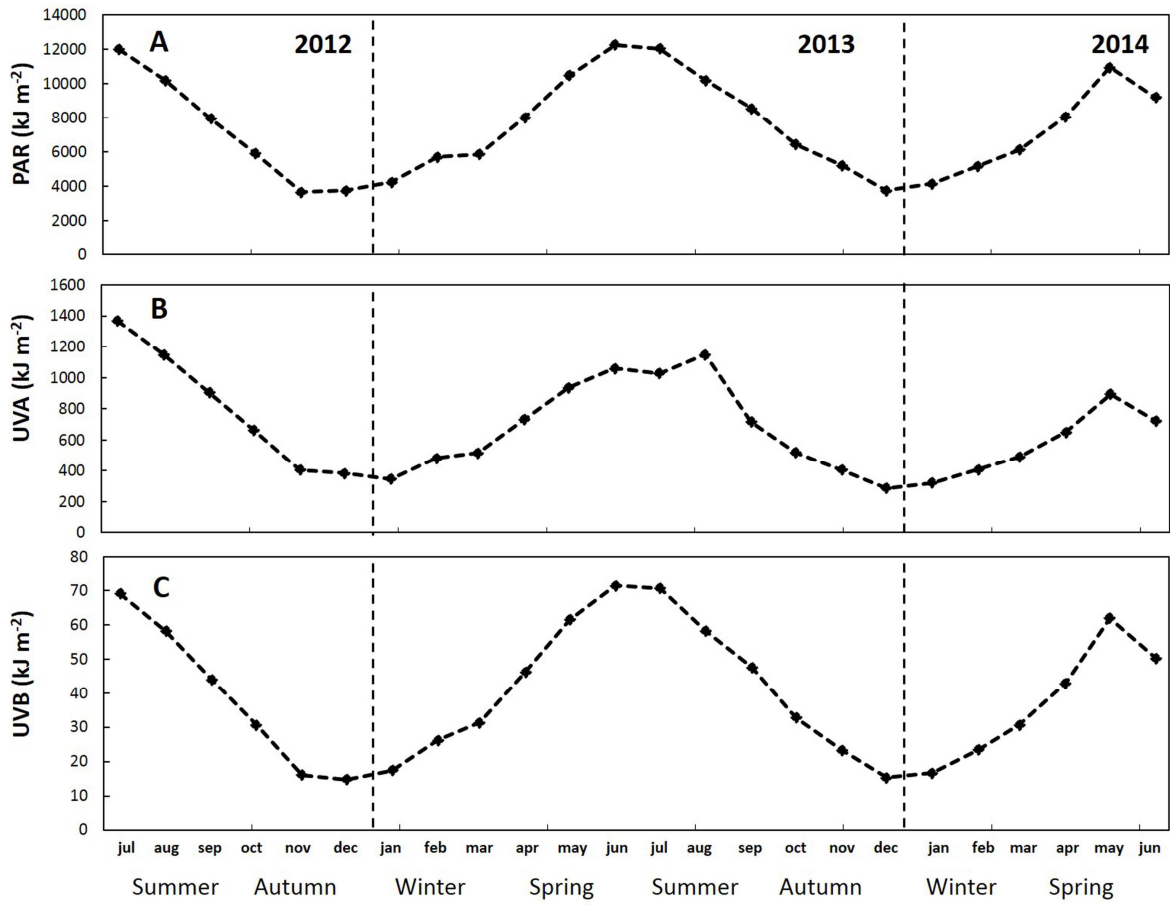
**Winter**

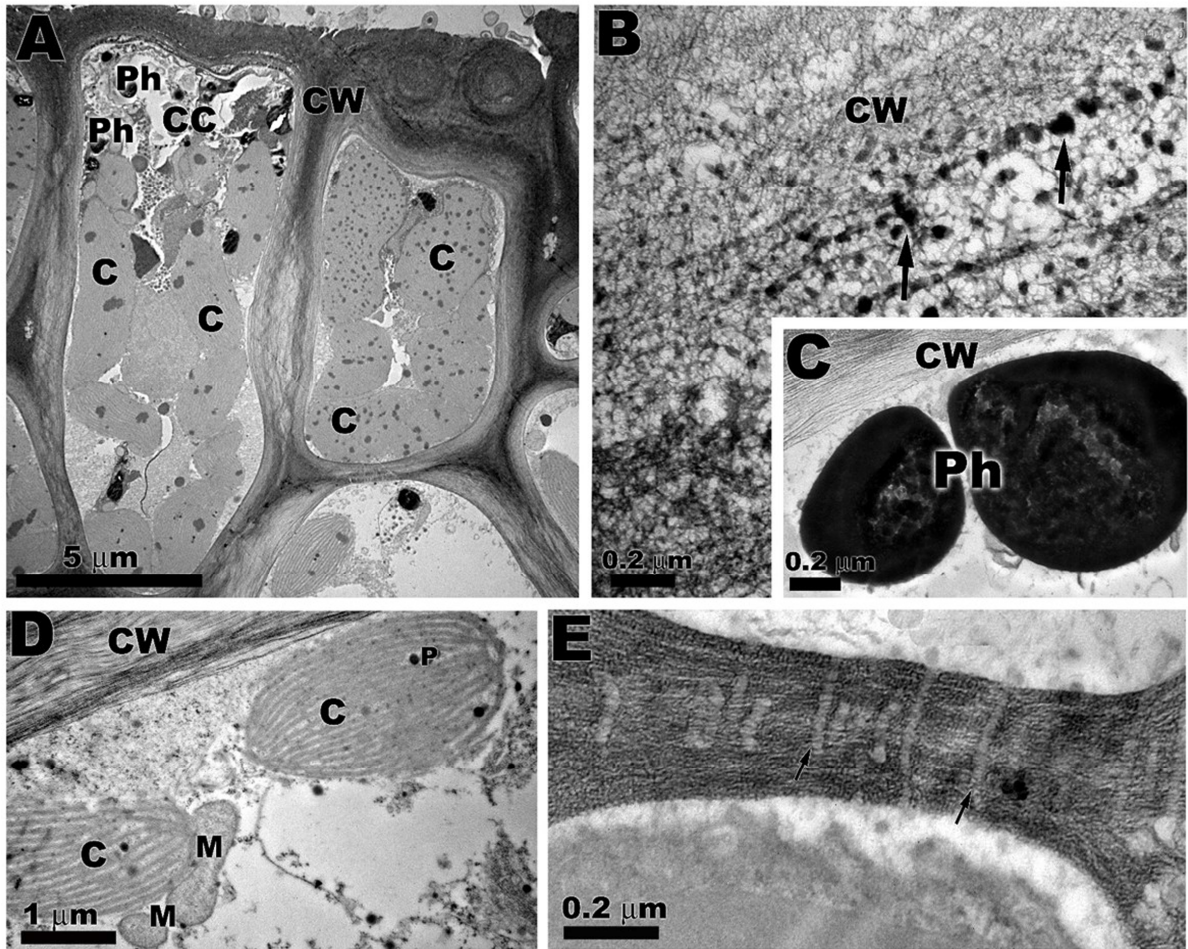


**Spring**

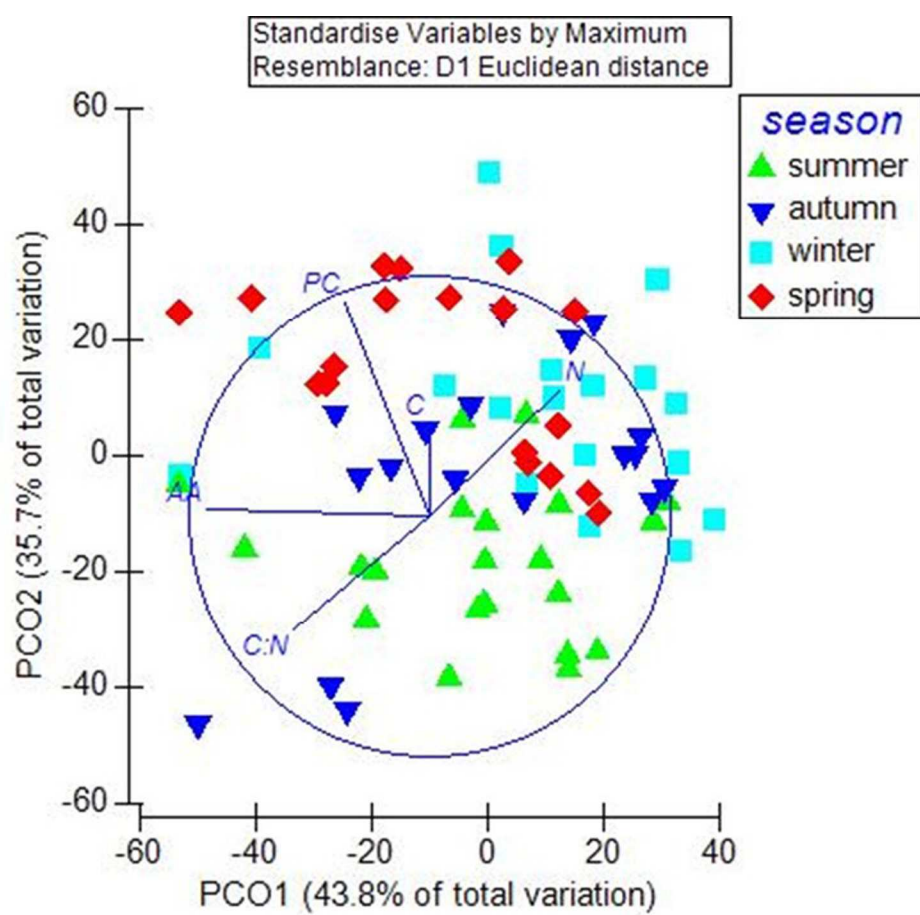


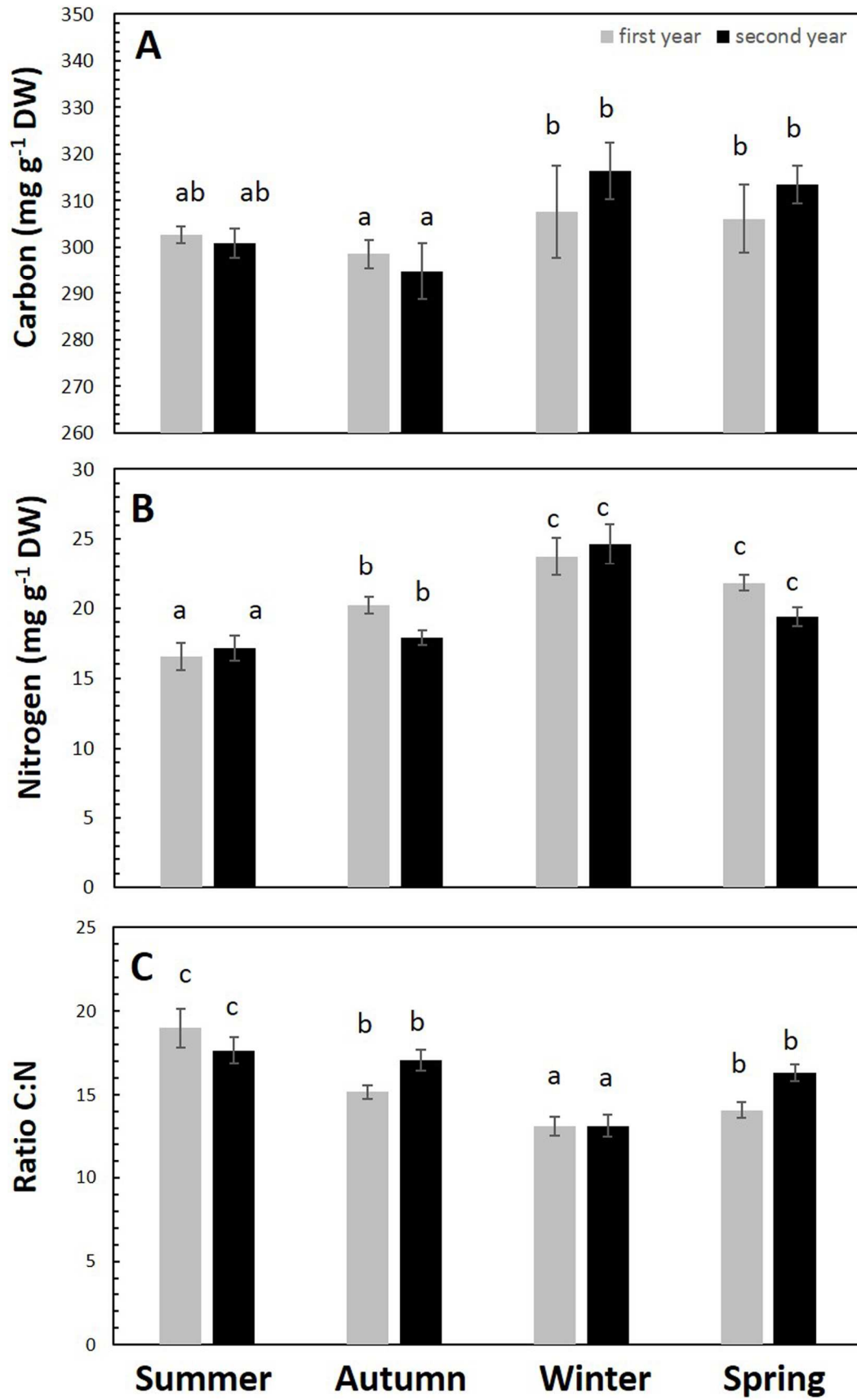


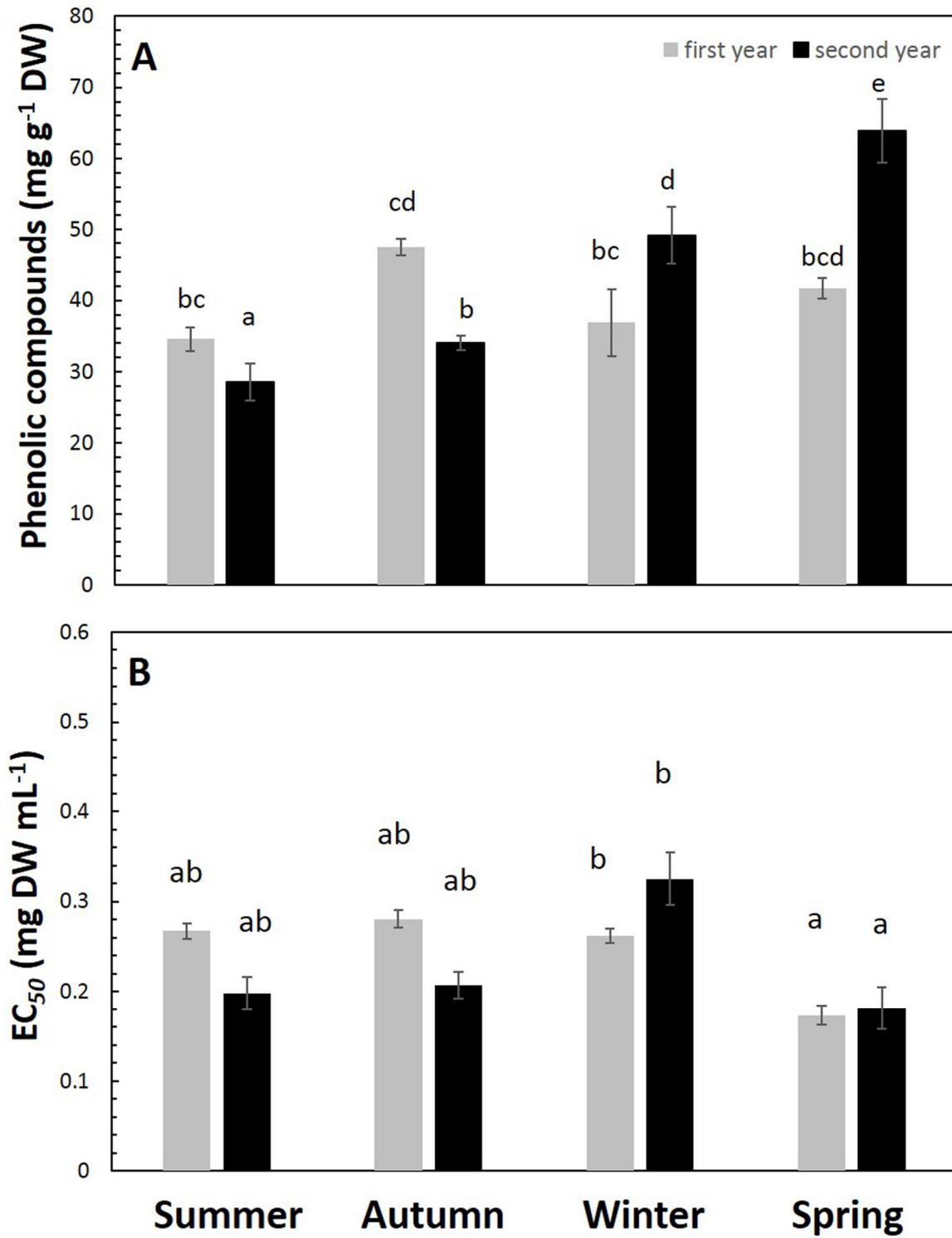




ACCEPTED







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

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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.