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Effects of heavy metals and light levels on the biosynthesis of carotenoids and fatty acids in the macroalgae *Gracilaria tenuistipitata* (var. *liui* Zhang & Xia)

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Abstract: We present here the effect of heavy metals and of different light intensities on the biosynthesis of fatty acids and pigments in the macroalga *Gracilaria tenuistipitata* (var. liui Zhang & Xia). In order to verify the fatty acid content, gas chromatography with flame ionization detection (GC-FID) was employed. Pigments (major carotenoids and chlorophyll-*a*) were monitored by liquid chromatography with diode array detection (HPLC-DAD). Cultures of G. tenuistipitata were exposed to cadmium (Cd^{2+}, 200 ppb) and copper (Cu^{2+}, 200 ppb), as well as to different light conditions (low light: 100 µmol.photons.m-2.s^{-1}, or high light: 1000 µmol.photons.m^{-2}.s^{-1}). Cd^{2+} and Cu^{2+} increased the saturated and monounsaturated fatty acid content [14:0, 16:0, 18:0, 18:1 (n-7) and 18:1 (n-9)] and all major pigments (violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll-*a* and β-carotene). Both heavy metals decreased the levels of polyunsaturated fatty acids (PUFA) [18:2 (n-6), 18:3 (n-6), 18:5 (n-4), 20:4 (n-6), 20:5 (n-3), 22:6 (n-3)]. *G. tenuistipitata* cultures were exposed to high light intensity for five days and no statistically significant differences were observed in the content of fatty acids. On the other hand, the levels of pigments rose markedly for chlorophyll-*a* and all of the carotenoids studied.

Article

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Introduction

Diverse environmental conditions can clearly interfere with the biosynthesis of natural products from algae (Pinto et al., 2003a). Variations in the light intensity, temperature, pH, UV radiation, content of macro- and micronutrients, sea tides and presence of pollutants have been described as natural and anthropogenic disturbances that can influence the physiology, composition and quantity of some marine macroalgal metabolites (Franklin & Forster, 1997; Hader & Figueroa, 1997; Hader et al., 1998; Pinto et al., 2003b; Cardozo et al., 2007; Gressler et al., 2011).

Fatty acids and carotenoids play an important role in the function and integrity of the chloroplast thylakoid membrane of photosynthetic organisms (Frank & Cogdell, 1996; Havaux, 1998; Sigaud-Kutner et al., 2002; Su et al., 2010). In fact, algal fatty acids (saturated, monounsaturated and polyunsaturated - PUFA) and carotenoids (carotenes and xanthophylls) vary their quality and quantity according to the environmental conditions (Barros et al., 2005; Britton, 1995; Cardozo et al., 2002; Collen et al., 2003; Colombo et al., 2006). The genera belonging to the Rhodophyta phylum are generally rich in PUFA (Khotimchenko et al., 2002; Li et al., 2002; Wahbeh, 1997). Rhodophyta species found in low temperature waters are usually richer in PUFA, with a higher n-3/n-6 fatty acid ratio, and possess a higher degree of total unsaturation. It has been reported that the genus *Gracilaria* contains mainly C 20:4 (n-6) and C 20:5 (n-3) and other saturated and monounsaturated fatty acids (Gressler et al., 2010). Heavy metals can be accumulated by some species of macroalgae and interfere with photosynthesis (Baumann et al., 2009; Gledhill et al., 1997). It was also reported that copper (Cu) and cadmium (Cd) induce oxidative stress in *G. tenuistipitata* (Collen et al., 2003).

Macroalgae biosynthesize a variety of

pigments, including several carotenoids that are usually found in terrestrial plants (*i.e.*, violaxanthin, antheraxanthin, zeaxanthin, lutein and β-carotene) as well as chlorophyll-*a* (Cardozo et al., 2007). Under culture conditions, light and heavy metals have been shown to induce the biosynthesis of carotenoids in *G. tenuistipitata*. Carnicas et al. (1999) described an oscillation of pigments in *G. tenuistipitata* when cultures were grown under two different irradiance levels. After addition of Cu^{2+} and Cd^{2+}, the content of β-carotene and lutein of this species increased during growth (Collen et al., 2003).

The purpose of the present investigation was to simulate two different environmental situations with cultures of *G. tenuistipitata* and to monitor the biosynthesis of fatty acids and pigments. The first experiment was carried out to mimic an area polluted with heavy metals (low amounts of Cd^{2+} and Cu^{2+} were added to the cultures). The second assay was designed to reproduce a natural condition of variable light intensity (cultures were grown under 100 or 1000 $\mu mol.photons.m^{-2}.s^{-1}$). Our findings indicate that both conditions interfere with the biosynthesis of fatty acids and pigments, maintaining *G. tenuistipitata* homeostasis and coping with oxidative stress.

Materials and Methods

Chemicals and reagents

The pigments violaxanthin, antheraxanthin and zeaxanthin were obtained from DHI, Water & Environment©, and lutein, β-carotene and chlorophyll-*a* from Sigma-Aldrich®. The fatty acid mixture was purchased from Sigma-Aldrich®. Merck (Darmstadt, Germany) supplied HPLC grade methanol and ethyl acetate. All other chemicals were of analytical grade.

Cultures

The marine red macroalga *G. tenuistipitata* Chang (var. *liui* Zhang & Xia) was grown at an irradiance of 100 $\mu mol.photons.m^{-2}.s^{-1}$ at 20 °C in seawater diluted to 20 ppt with distilled water, bubbled with air, with a light:dark (12 h:12 h) regime before the start of the experiments. The algae were fertilized according to Haglund et al. (1996), a modification of Provasoli et al. (1957), and fertilized the day before the experiment; no nutrients were added during the experiment. The seawater was changed at the start of the experiment. Heavy metals were added as solutions of $CdCl_2$ (Cd^{2+}, 200 ppb) and $CuSO_4.5H_2O$ (Cu^{2+}, 200 ppb). During the experiments, the conditions were identical except for the light level; two irradiances were used, 100 (control, low light intensity, L-) and 1000 $\mu mol.photons.m^{-2}.s^{-1}$ (high light intensity, L+). The initial density of algae in the experiments was 1 g fresh weight (FW) per L. For the control and heavy metals experiments, harvesting was done 6 h after the start of the light period. For the high-light experiments, harvesting was performed 6 and 18 h after the start of the light period (for both control L- and L+). Samples were frozen in liquid nitrogen and kept at -70 °C until analyzed.

Pigment analyses

Chlorophyll-*a* (Chl-a), β-carotene, violaxanthin, antheraxanthin, lutein and zeaxanthin were extracted following the procedure described by Carnicas et al. (1999) with a few modifications. Briefly, 2 mL of dimethylformamide were added to 200 mg dried algae (lyophilized). After incubation in an ultrasonic bath (10 min), 1 mL of methanol/ethylacetate (MeOH/EtOAc, 1:1) was added and the samples were filtered through 0.22 µm Millipore® membranes. Aliquots of the extracts (100 µL) were injected onto the HPLC. The analysis was performed on a Shimadzu HPLC system consisting of two LC-10AD pumps fitted with an auto-injector (SIL-10ADVP), a photodiode array detector (SPD-M10AVP) set at 445 nm, and a system-controller (SCL-10AVP). Data were stored in Class-VP 5.032 software. A Luna RP-18 column (250 mm x 4.6 mm, particle size 5 µm, Phenomenex®), with a pre-column guard (particle size 5 µm, RP-18 Phenomenex®) was used for pigment separation. The flow rate was 1.0 $mL.min^{-1}$ and MeOH and EtOAc were HPLC grade (Merck®). The gradient was achieved with 70% MeOH and EtOAc; for the first 6 min, the gradient was 25-27% EtOAc followed by 4 min at 27% EtOAC and then increased to 75% EtOAc over 5 min and maintained constant at 75% EtOAc for 12 min. Pigments were identified by comparing retention times and absorption spectra with authentic standards.

Fatty acid analyses

Fatty acid methyl esters (FAME) were obtained by direct transesterification of lyophilized samples of *G. tenuistipitata*, as described by Carvalho & Malcata (2005a, b). Acetyl chloride was used as the derivatization agent and heptadecanoic acid as internal standard. FAMEs were analyzed with a gas chromatograph (Hewlett Packard 5890), equipped with a flame ionization detector and a polar, 60-m fused silica Supelcowax10 (Supelco®) capillary column. The oven temperature was programmed to increase from 170 to 220 °C at a rate of 1 oC/min; the injector and detector temperatures were 250 and 270 °C, respectively; helium was used as the carrier gas. Pure standards of the free fatty acids (Sigma®) were used for fatty acid

identification, which was based on comparison of the peak retention times of the samples and standards under similar elution conditions. Peak areas were quantified by automatic integration (Hewlett Packard 3395) and calculations were performed according to the AOCS Official Method Ce lb-89 (Firestone, 1994). Fatty acids were named using the code i:j (*n-k*), where i indicates the total number of carbon atoms, j the number of double bonds, and k the position of the last double bond counted from the terminal methyl group.

Statistical analyses

Data were expressed as average values±SD (standard deviation). The results were tested for significance at the 0.05 level by comparing the average value obtained for the control (three replicates) with those of the experiments performed with varying light intensity (three replicates) or in the presence of heavy metals (three replicates) employing one-way analysis of variance and the Student-Newman-Keuls multiple comparison test.

Results and Discussion

The major fatty acids found in *G. tenuistipitata* [14:0, 16:0, 18:0, 18:1 (n-7), 18:1 (n-9), 18:2 (n-6), 18:3 (n-6), 18:5 (n-4), 20:4 (n-6), 20:5 (n-3), 22:6 (n-3)] were similar to those already reported for other *Gracilari*a and red algae species (Colombo et al., 2006; Gressler et al., 2010; Khotimchenko et al., 2002; Li et al., 2002; Moore, 2006; Wahbeh, 1997). When cultures were exposed to Cu^{2+} and Cd^{2+}, the amount of saturated and monounsaturated fatty acids [14:0, 16:0, 18:0, 18:1 (n-7), 18:1 (n-9)] increased (Figure 1A). In contrast, the levels of PUFA [18:2 (n-6), 18:3 (n-6), 18:5 (n-4), 20:4 (n-6), 20:5 (n-3), 22:6 (n-3)] diminished, the decrease being more significant for the treatment with Cd^{2+} (Figure 1B). No significant variation of the fatty acids was found when cultures were submitted to high light intensity (data not shown).

Heavy metals can change the lipid and fatty acid composition of algae (Rocchetta et al., 2006; Vavilin et al., 1998). The mechanisms involve mostly oxidative stress and the production of reactive oxygen/nitrogen species that lead to oxidation of lipids (Pinto et al., 2003b). As expected and supported by other experiments with cultures of algae (Collen et al., 2003; Okamoto et al., 2001; Rocchetta et al., 2006), PUFA are the fatty acids most affected and their levels are reduced in the presence of heavy metals. In our experiments, Cd^{2+} proved to be more toxic than Cu^{2+} and significantly decreased the concentration of PUFA.

The evolution and physiological aspects of thylakoid membranes in plants and algae are not completely elucidated. The profile of fatty acids and carotenoids may change according to the species and the environmental conditions (Benning, 2008; Triantaphylides & Havaux, 2009). Carotenoids play an important role in membrane stability, as well as being natural antioxidants (Britton, 1995; Havaux, 1998; Havaux & Niyogi, 1999; Guaratini et al., 2005). As shown in Figure 2, the quantities of all carotenoids and of chlorophyll-*a* markedly increased in the presence of Cu^{2+} and Cd^{2+}. However, no difference was observed between Cu^{2+} and Cd^{2+} treatments.

Xanthophylls have a particular function in photosynthetic organisms. Terrestrial plants and most

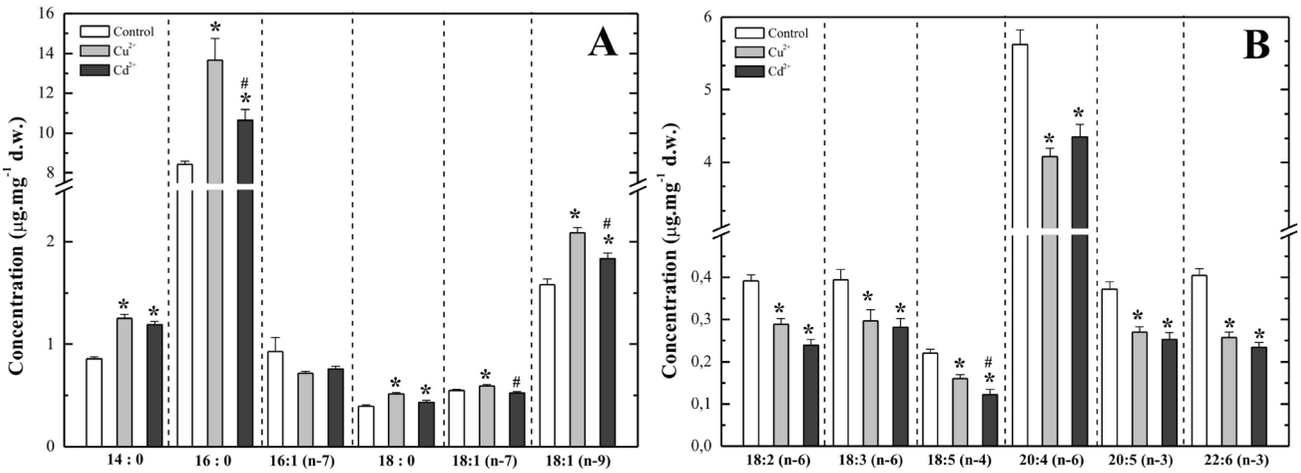

Figure 1. Content of fatty acids in cultures of *G. tenuistipitata* exposed to Cu^{2+} and Cd^{2+} (200 ppb). A. corresponds to the total amount of saturated and monounsaturated fatty acids and B. represents the concentration of PUFA. Results were obtained by GC-FID as described in the section "Fatty acid analyses". Values are presented as the average±SD of three replicates compared to the control (*p<0.05) or between Cu^{2+} and Cd^{2+} treatments (#p<0.05). Comparison was performed by ANOVA, with the Student-Newman-Keuls multiple comparison test.

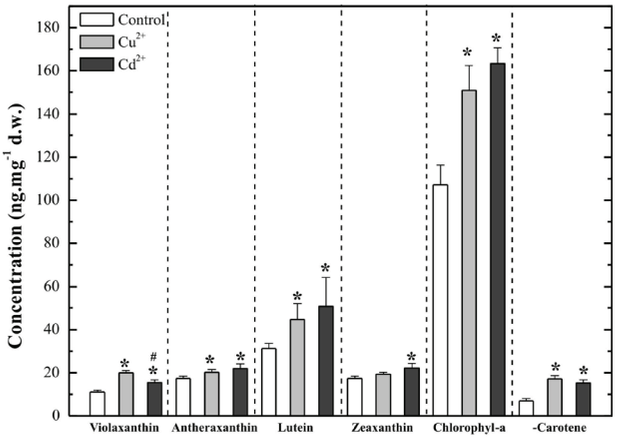

Figure 2. Content of pigments in cultures of *G. tenuistipitata* exposed to Cu^{2+} and Cd^{2+} (200 ppb). Results were obtained by HPLC-DAD as described in the section "Pigment analyses". Values are presented as the average±SD of three replicates compared to the control (*p<0.05) or between Cu^{2+} and Cd^{2+} treatments (#p<0.05). Comparison was performed by ANOVA, with the Student-Newman-Keuls multiple comparison test.

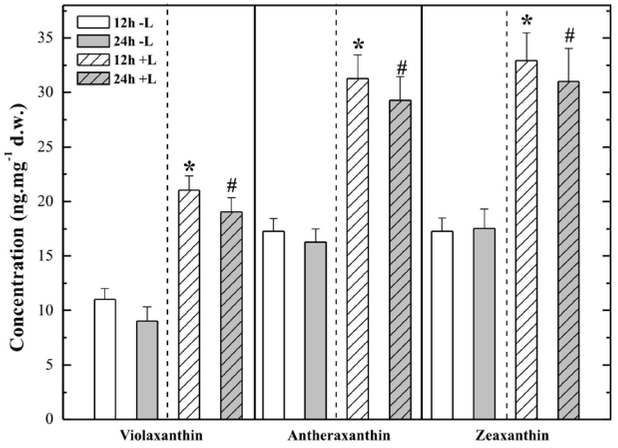

Figure 3. Levels of pigments involved in the Xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) in cultures of *G. tenuistipitata* under two different light intensities (100 µmol. photons.m^{-2}.s^{-1} - control, low light intensity: L- and 1000 µmol. photons.m^{-2}.s^{-1}, high light intensity: L+) and at two periods (12 h, middle of the day phase, and 24 h, middle of the dark phase). Results were obtained by HPLC-DAD as described in the section "Pigment analyses". Values are presented as the average±SD of three replicates compared to the control (*p<0.05) or between the 12 h and 24 h periods (#p<0.05). Comparison was performed by ANOVA, with the Student-Newman-Keuls multiple comparison test.

all macroalgae possess the so-called xanthophyll cycle. Under certain stressful conditions, including high light incidence, plants can dissipate the excess energy in photosynthesis via a cyclic reaction involving two successive de-epoxidations of violaxanthin to zeaxanthin, with antheraxanthin as an intermediate (DemmigAdams & Adams, 1996; Eskling et al., 1997; Havaux & Niyogi, 1999). As mentioned above, low and high light irradiances (L- and L+: 100 and 1000 µmol.photons.m^{-2}.s^{-1}, respectively) had no effect on the biosynthesis of fatty acids in *G. tenuistipitata*. Nevertheless, the quantity of carotenoids related to the xanthophyll cycle showed a singular pattern, with zeaxanthin, anteroxanthin and violaxanthin increasing significantly under L+ (Figures 3). Our results corroborate the findings of Carnicas et al. (1999), indicating that the xanthophyll cycle may play an important role under conditions of high light exposure.

The other pigments had comparable profiles; the content of lutein, chlorophyll-*a* and β-carotene increased under L+ (Figure 4). These results are related to previous experiments performed by our group (Collen et al., 2003), where the concentrations of lutein and β-carotene in cultures of *G. tenuistipitata* were found to increase under an irradiation intensity of 250 µmol.photons.m^{-2}.s^{-1}. In addition, in the control (L-), no difference was noted when samples were collected after 6 or 18 h (middle of the day phase at 12 h or middle of the dark phase at 24 h, respectively). On the contrary, when compared to the control, L+ experiments presented higher amounts of pigments at both 12 h or 24 h harvest times (Figures 3 and 4).

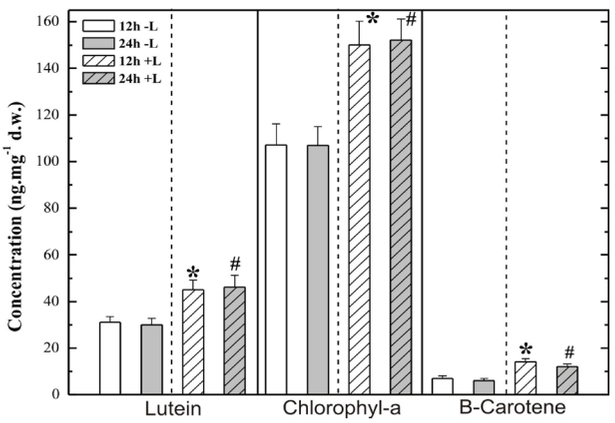

Figure 4. Levels of pigments (lutein, chlorophyll-*a* and β-carotene) in cultures of *G. tenuistipitata* under two different light intensities (100 µmol.photons.m^{-2}.s^{-1} - control, low light intensity: L- and 1000 µmol.photons.m^{-2}.s^{-1}, high light intensity: L+) and at two periods (12 h, middle of the day phase, and 24 h, middle of the dark phase). Results were obtained by HPLC-DAD as described in the section "Pigment analyses". Values are presented as the average±SD of three replicates compared to the control (*p<0.05) or between the 12 h and 24 h periods (#p<0.05). Comparison was performed by ANOVA, with the Student-Newman-Keuls multiple comparison test.

Conclusions

Our results point out that heavy metals and different light conditions modify the biosynthesis of

fatty acids and pigments, showing that cultures of *G. tenuistipitata* can modify its physiology under some environmental stressful conditions.

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