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 macroalgae *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 (Cd2+, 200 ppb) and copper (Cu2+, 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). Cd2+ and Cu2+ 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), 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.

Keywords: *Gracilaria tenuistipitata* carotenoids xanthophylls heavy metals fatty acids light intensity

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). Macroalgaes 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 Cu²⁺ and Cd²⁺, 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 Cd²⁺ and Cu²⁺) 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 µmol.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 µmol.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 CdCl₂ (Cd²⁺, 200 ppb) and CuSO₄·5H₂O (Cu²⁺, 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 µmol.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, antheroxanthin, 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 °C/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 Gracilaria 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 Cu2+ 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 Cd2+ (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, Cd2+ proved to be more toxic than Cu2+ 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-β markedly increased in the presence of Cu2+ and Cd2+. However, no difference was observed between Cu2+ and Cd2+ treatments.

Xanthophylls have a particular function in photosynthetic organisms. Terrestrial plants and most
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Figure 2. Content of pigments in cultures of G. tenuistipitata exposed to Cu²⁺ and Cd²⁺ (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 Cu²⁺ and Cd²⁺ 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.

Figure 4. Levels of pigments (lutein, chlorophyll-α 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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