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Absorption of metals and characterization of chemical elements present in three species of *Gracilaria* (Gracilariaceae) Greville: a genus of economical importance

Angela P. Tonon,[*1] Mariana C. Oliveira,[2] Eliane M. Soriano,[3] Pio Colepicolo[1]

[1]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Brazil,*
[2]*Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, Brazil.*
[3]*Departamento de Oceanografia e Limnologia, Universidade do Rio Grande do Norte, Brazil.*

Article

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Abstract: *Gracilaria Greville* is a genus of seaweed that is economically explored by the cosmetic, pharmaceutical and food industries. One of the biggest problems associated with growing *Gracilaria* is the discharge of heavy metals into the marine environment. The absorption of heavy metals was investigated with the macroalga *Gracilaria tenuistipitata* Zhang et Xia, cultivated in a medium containing copper (Cu) and cadmium (Cd). In biological samples, EC50 concentrations of 1 ppm for cadmium and 0.95 ppm for copper were used. These concentrations were based on seaweed growth curves obtained over a period of six days in previous studies. ICP-AES was used to determine the amount of metal that seaweeds absorbed during this period. *G. tenuistipitata* was able to bioaccumulate both metals, about 17% of copper and 9% of cadmium. Basal natural levels of Cu were found in control seaweeds and in *G. tenuistipitata* exposed to Cd. In addition, the repertoire of other important chemical elements, as well as their concentrations, was determined for *G. tenuistipitata* and two other important seaweeds, *G. birdiae* Plastino & Oliveira and *G. domingensis* (Kützing) Sonder ex Dickie, collected in natural environments on the Brazilian shore.

Keywords:
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Introduction

The genus *Gracilaria* (Greville), a red macroalgae of the family Gracilariaceae (Rhodophyta) is extensively used in industry, due to its production of secondary metabolites of economic impact (Lee et al., 1999). This genus has high commercial value as a source of agar, a sulphated hydrocolloid (McHugh, 1991; Falcão et al., 2008). In addition, many secondary metabolic compounds have been isolated from *Gracilaria* and applied as antitumorals, antimicrobials and antioxidants. The majority of these are terpenes, but several fatty acids are also common as well as nitrogenous compounds (Van Alstyne & Paul, 1988; Cardozo et al., 2007; Boobathy et al., 2010; Zandi et al., 2010; Jain et al., 2010; Falcão et al., 2010). One of the greatest difficulties in *Gracilaria* cultivation is the gradual increase in the discharge of heavy metals and other pollutants into the environment. Marine organisms, including phytoplankton and macroalgae, are directly exposed to these chemical elements, which, due to their various degrees of toxicity, constitute an impediment to the development of the organisms, leading to a decrease in both growth and biodiversity in the environment (Pinto et al., 2003; Torres et al., 2008).

Brazil has several natural seaweed beds and various species of *Gracilaria* of economic importance. This, together with an extensive coastline, provides opportunities for their cultivation. Therefore, a screening of the concentrations of various chemical elements was undertaken for *G. birdiae* Plastino & Oliveira and *G. domingensis* (Kützing) Sonder ex Dickie in their natural environments. Because these species are already being studied and commercialized, they are worthy of special note.

In the present work, we estimated the absorption of copper (Cu) and cadmium (Cd) and the content of chemical elements in three species of *Gracilaria: Gracilaria tenuistipitata*, cultivated in our laboratory and exposed to Cu and Cd, and *G. birdiae* and *G. domingensis* collected in their natural environments.

Materials and Methods

Growth conditions

Specimens of *G. tenuitipitata* were cultivated in seawater enriched with von Stosch solution (Edwards, 1970) at a temperature of 23 ºC, with a plant density of 10 $g.L^{-1}$, under a light/dark cycle of 12-12 h at an irradiance of 100 µmol $photon.m^{-2}$ s^{-1}, provided by a fluorescent lamp. The culture medium was renewed every six days and presented a pH 7.5.

EC50 determination for Cu and Cd

The EC50 (Median Effective Concentration) was used in the toxicity-bio assays of these two metals. The methodology employed for seaweed exposure to metals and for the estimation of toxicological parameters was performed according to international protocol (EPA, OECD). The EC50 values were 0.95 ppm for Cu and 1 ppm for Cd. These values were encountered based on previous studies of the growth curve of these seaweeds during six days of treatment (Neto, 2008).

Experimental conditions

In order to evaluate the effects of Cu and Cd on *G. tenuistipitata*, the starting point was six days of exposure to EC50 of the metals. Samples of seaweed were added to the culture medium (von Stosch medium, 60% of filtered and sterilized sea water and 40% of MilliQ water), together with concentration of each metal equivalent to its EC50. The salts used were $CdCl_2$ and $CuSO_4$. The amount of 10 g of seaweed was inoculated in 2 L of culture medium with the metal solution already added. The two treatments and the control were performed in triplicate. The conditions of light and temperature were similar to the conditions used in the laboratory culture.

After six days of exposure to the metals, triplicate samples of the seaweeds were collected for each of the three experimental conditions: control (normal culture conditions), exposure to Cu and exposure to Cd. In addition, three culture medium controls were used; thus, culture medium diluted in sea water was measured 1) before the addition of the metals, 2) after the addition of the metals, and 3) after six days of exposure of the algae to the metals. Thus, it was possible to measure the amount of Cd and Cu in the culture medium before and after adding the metals and at the end of the exposure of the seaweeds. Aliquots were analyzed in triplicate for each of the controls (1, 2 and 3, above).

Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

Metal analysis was performed with an atomic emission spectrophotometric system with an inductively linked plasma source (ICP-AES, Genesis, SOP model). Five standard were used to so obtain complete adjustment of the apparatus. The standard curves were obtained using concentrations of 0, 1, 2, 5 and 10 ppm for both Cu and Cd to convert from counts [cps] to concentration [ppm] (Figure 1). Data were analyzed by using *Smart Analyzer* software.

The other chemical elements Al, Ca, Fe, Mg, P, Si, Mn, B, K, Na, Ba, Ni, Zn, Sr, Cr, Co and As were also analyzed by ICP-AES. The same procedure mentioned above was used to calibrate the response for each element. Two species of Brazilian *Gracilaria* (*G. birdiae* and *G. domingensis*) were used for comparison

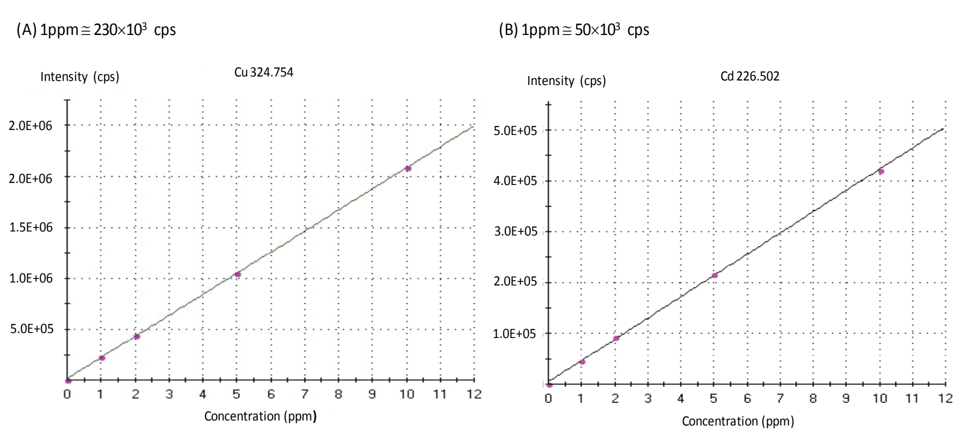

Figure 1. Standard curves for copper (A) and cadmium (B), used to convert between counts per second [cps] and concentration [ppm].

with *G. tenuistipitata*. The Brazilian species were collected in their natural coastal environments at Cotovelo (05°56'S-35°09'W) and Rio do Fogo beaches (05°16'S-35°22'W), Rio Grande do Norte (RN).

Results

Absorption of Cu and Cd

Figure 2 shows the atomic emission spectrum (counts per second [cps] *versus* wavelength λ [nm]) for Cu. Spectra 1 and 2 represent, respectively, the 1 ppm Cu standard and the seaweed exposed to Cu. The basal concentrations of Cu in seaweed exposed to Cd and in the control are indicated by spectra 3 and 4, respectively. In the atomic emission spectrum for Cd (Figure 3), no other representative emissions were detected in spectra 1 or 2 besides those of Cd in the 1 ppm standard and in algae exposed to Cd, respectively.

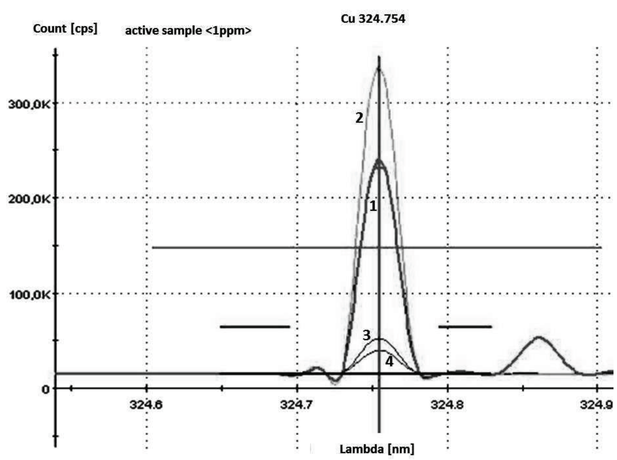

Figure 2. Atomic emission spectra for copper. Spectrum 1 represents the standard Cu concentration of 1 ppm. Spectrum 2 represents the concentration of copper in seaweeds exposed to copper. Spectrum 3 represents the basal concentration of copper in seaweeds exposed to cadmium. Spectrum 4 represents the concentration of copper in unexposed seaweed.

From the analysis of the ICP-AES results, the means and standard deviations of the concentrations of metals absorbed by seaweeds treated with Cu, Cd and control were obtained from experiments performed in triplicate. In the case of seaweed exposed to Cu, concentrations of Cd were also measured to serve as a control in the experiment. The same was done for seaweed exposed to Cd, in which Cu was measured. The concentrations of both metals (Cu and Cd) were also measured for the control alga. The data presented in Table 1, based on the calibration curves in Figure 1, were extracted from an analysis of the ICP-AES spectra. The amount of Cu absorbed by the seaweed was obtained by subtracting the basal amount from the total amount for a 10 g sample, resulting in 0.325 mg (Table 1). This result is in accord with the value obtained from the difference between the initial and final amounts of Cu in the medium (0.34 mg). The same analysis, carried out for Cd, gave an absorbed amount of 0.204 mg.

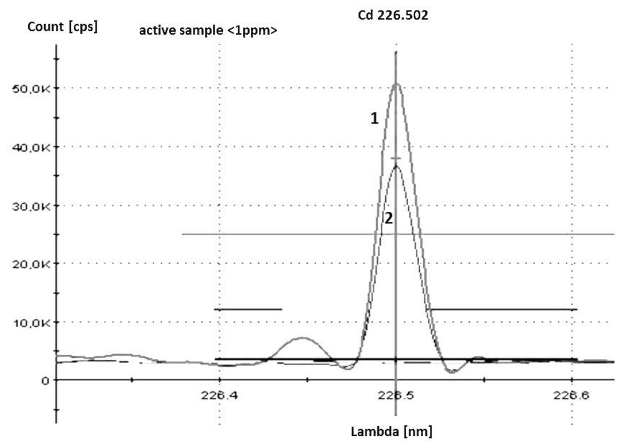

Figure 3. Atomic emission spectrum of cadmium. Spectrum 1 represents the standard concentration of 1 ppm and spectrum 2 the concentration of cadmium in seaweeds exposed to cadmium.

Figure 4 shows the comparison between the amount of metals found in the culture medium before and after exposure of the algae, as well as the values of the amounts absorbed by the alga (calculated from the analysis of the ICP-AES results, Table 1). The percentage of metal absorption is also shown. Note that *G. tenuistipitata* is able to absorb about two-fold more Cu as compared with Cd.

Elemental composition of three species of Gracilaria

The results of the screening for chemical elements detected by ICP-AES can be observed in Figure 5; the mean and standard deviation of each chemical element are shown in Table 2. For the three *Gracilaria*

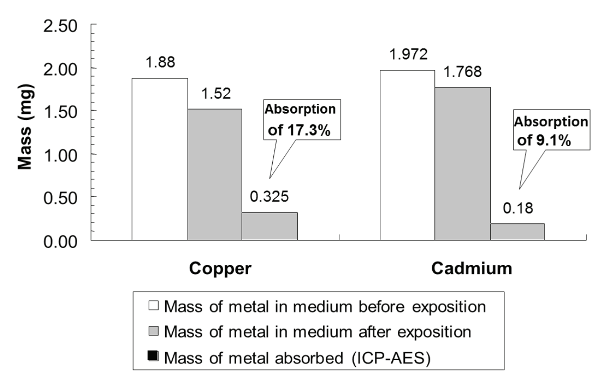

Figure 4. Mass balance between the amounts of metal analyzed.

Table 1. Levels of copper and cadmium in *G. tenuistipitata* and in the culture medium.

Samples	Cu			Cd		
	Peak [Kps]	Concentration [ppm]	Amount [mg]	Peak [Kps]	Concentration [ppm]	Amount [mg]
Seaweed exposed to Cu	330±6	1.43±0.03	0.0358±0.0007	-	<0.01	-
Seaweed exposed to Cd	50±1	0.22±0.01	0.0060±0.0002	36±4	0.72±0.09	0.018±0.002
Unexposed Seaweed	30±1	0.130±0.006	0.0033±0.0001	-	<0.01	-
Initial medium+Cu	-	0.94	1.88	-	<0.01	-
Final medium+Cu	-	0.77	1.52	-	<0.01	-
Initial medium+Cd	-	<0.01	-	-	0.986	1.972
Final medium+Cd	-	<0.01	-	-	0.884	1.768

species, the concentration levels were different for some of the elements analyzed. For example, the concentration of aluminum (Al) in *G. tenuistipitata* was less than half of that in the other two species. The concentration of calcium (Ca) was two-fold higher in *G. domingensis* than in the other two species, while the concentration of manganese (Mn) was two-fold higher in *G. birdiae* than in *G. tenuistipitata* and *G. domingensis*. Cu was found in basal concentrations in all three species analyzed, whereas Cd was absent in all seaweed samples. Even though the elements Ba, Ni, Zn, Sr, Cr, Co, As and Cd were analyzed, none were detected in the three species.

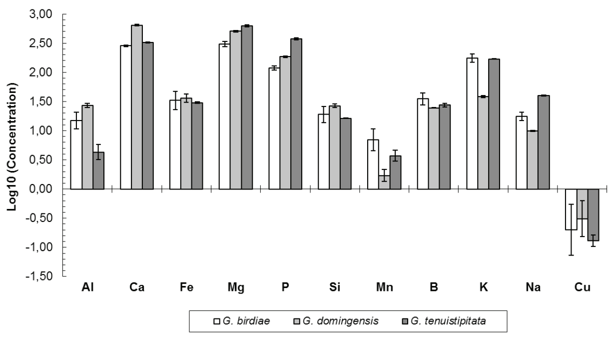

Figure 5. Concentrations of chemical elements encountered in the seaweeds *G. birdiae, G. domingensis, G. tenuistipitata* by ICP-AES analysis (from the data of Table 2), presented on a logarithmic scale to facilitate graphical comparison of the results.

Discussion

After exposing *G. tenuistipitata* to Cd and Cu during six days, the bioaccumulation these metals by the alga became evident. *G. tenuistipitata* was able to accumulate higher concentrations of Cu as compared to Cd. A plausible explanation for this difference might be the capacity of Cu to be transported into the cell. Since it is essential for seaweed metabolism, there might possibly exist specific transporters for this metal. In *Arabidopsis thaliana*, five members of a family of Cu transporters (COPT1-5) have been described and, in *Saccharomyces cerevisiae*, a gene (CTR1) that codifies proteins of the plasmatic membrane that transports Cu into the cell has been characterized (Dancis et al., 1994; Grotz, 1998; Himelblau & Amasino, 2000; Sancenon et al., 2003; Markossian & Kurganov, 2003). Basal natural concentrations of Cu were present in the control seaweeds (with and without Cd). Even without deliberate exposure. Cu is an essential metal for *G. tenuistipitata*, both as an enzymatic co-factor (amine oxidase and cytocrome c oxidase) and as an electron carrier in the process of photosynthesis (plastocyanin) (Gledhill et al., 1997; Collen et al., 2003). It is noteworthy that Cu is not a component of the von Stosch medium, i.e., the basal concentrations of this metal presumable reflect accumulation from sea water. Nonetheless, studies with terrestrial plants and green unicellular algae demonstrated that Cu in excess becomes toxic to these organisms, leading to production of Reactive Oxygen Species (ROS), which are detrimental to plant-growth (Charrier et al., 2008). Therefore, upon exposition of *G. tenuistipitata* to Cu in excess, cellular, biochemical and molecular responses are expected, leading to acclimatization of the seaweed to this condition.

In contrast, Cd has no known biological function in the metabolism of *G. tenuistipitata.* Stohs & Bagchi (1995) demonstrated that there is evidence of its transport into the cells. In terrestrial plants, it has been demonstrated that Cd competes for divalent ion carriers and can be transported with protons and type P ATPases. Studies on the biological effects of Cd are scarce (Hirschi et al., 2000). Stohs & Bagchi (1995) suggest that Cd ions dislocate zinc and iron from proteins. The liberation of iron ions might then induce the Fenton reaction, generating ROS and leading to total oxidative stress, with deleterious consequences for seaweed growth. Many toxic heavy metals, such as Cd, mercury (Hg) and lead (Pb), are able to compete for essential metal carriers during absorption (Guerrinot,

Table 2. Concentration (in ppm) of chemical elements measured by ICP-AES for the three species of algae.

Chemical element	*Gracilaria birdiae*	*Gracilaria domingensis*	*Gracilaria tenuistipitata*
Al	15±5	27.2±2.3	4.3±1.3
Ca	284±11	645±14.5	327±6
Fe	33±12	36±6	30.1±1.0
Mg	305±30	505±18	626±29
P	118±10	186±6	376±17
Si	19±6	26.7±1,8	16.31±0.18
Mn	7±3	1.7±0.4	3.7±0.8
B	35±8	24.62±0.29	27.4±1.9
K	177±29	38.4±1.5	168.8±1.5
Na	17.6±2.9	9.87±0.25	39.7±0.7
Cu	0,20±0,6	0.31±0.22	0.13±0.03
Cd	< 0.01	< 0.01	< 0.01

2000). Nevertheless, our results indicate that *G. tenuistipitata,* is a metal bio-accumulating organism.

The differences between the three species in the concentrations of the various elements in Table 2, may be due to physiological, biochemical or genetic differences between the seaweeds or to different acclimatization events that occurred in their environments or microenvironments (Figure 5). For example, Mn was present in a higher concentration in *G. birdiae* than in the other two species. Ca and K concentrations were higher in *G. domingensis* than in *G. tenuistipitata* and *G. birdae.* These results are relevant for future studies of the cultivation of macroalgae, both in tanks and the open sea, and for ecological, physiological, biochemical and molecular studies of bioremediation and bioprospection. The ICP-AES technique proved to be efficient for determining trace and ultra-trace amounts of metals, making it possible to compare the repertory of chemical elements necessary for the survival and growth of the three algal species investigated.

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***Correspondence**

Angela P. Tonon
Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo
Av. Prof. Lineu Prestes, 748, Butantã, 05508-900 São Paulo-SP, Brazil
angelapt@usp.br
Tel. +55 11 3091 3810