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ANTIOXIDANT RESPONSES IN *SCYTOSIPHON LOMENTARIA* (PHAEOPHYCEAE) INHABITING COPPER-ENRICHED COASTAL ENVIRONMENTS[1]

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***Scytosiphon lomentaria* (Lingb.) Link. (Phaeophyceae) is one of the two dominant seaweeds in a coastal area of northern Chile affected by copper mine wastes, where the concentration of copper in water and algal tissues remains higher than in non-impacted sites. Copper-loaded plants develop oxidative stress, as demonstrated by the increased levels of reactive oxygen species and lipoperoxides. This stress was associated with 1) an enhanced activity of the antioxidant enzymes catalase, glutathione peroxidase, ascorbate peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase and 2) an inhibition of the glutathione reductase activity. Furthermore, stressed plants showed a decrease in glutathione and phenolic compounds levels and an increase in total ascorbate. Reciprocal transplants revealed that plants rapidly adjusted their antioxidant system in response to the conditions of the receiving site. In individuals transplanted from the copper-enriched environment to the control site, normal levels of lipoperoxides and antioxidant compounds were restored in 48 h and antioxidant enzymes recovered their basal activities in 96 h. Individuals transplanted from the control site to the copper-enriched area adjusted their antioxidant compounds and antioxidant enzymes within 48 h and 96 h, respectively, and reached the functional status of the local plants. We conclude that *S. lomentaria* inhabiting the copper-enriched area buffered oxidative stress by a simultaneous involvement of antioxidant enzymes and water-soluble antioxidant compounds. These antioxidant responses were rapid and reversible, suggesting that copper resistance in *S. lomentaria* is a constitutive trait and that copper enrichment of the area did not result in a locally adapted copper-tolerant ecotype.**

Key index words: **adaptation; antioxidant metabolism; copper stress and resistance; physiological plasticity; *Scytosiphon lomentaria***

Abbreviations: **AP, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DCF, 2,4-dichlorofluoresceine; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GP, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHAR, monodehydroascorbate reductase; ROS, reactive oxygen species; U, micromoles of substrate consumed per min**

Oxidative stress is a physiological stage characterized by a net increase in reactive oxygen species (ROS) that results from an insufficient scavenging capacity of the antioxidant defenses (Baker and Orlandi 1995, Mittler 2002). Reactive oxygen species are produced directly by the excitation of O_2 and the subsequent formation of singlet oxygen or by the transfer of one, two, or three electrons to O_2, which results in the formation of superoxide radicals, hydrogen peroxide or hydroxyl radicals, respectively (Baker and Orlandi 1995). It is important to consider that ROS are the normal byproducts of a number of metabolic pathways, including photosynthesis (Asada 1999) and respiration (Dat et al. 2003). Responses of organisms to oxidative stress include the use of antioxidant enzymes, water-soluble antioxidant compounds, and lipid-soluble antioxidant molecules (Foyer et al. 1997, Noctor and Foyer 1998). Thus, when the production of ROS exceeds the scavenging capacity of the antioxidant defenses, oxidation of proteins, lipids, polysaccharides, and nucleic acids takes place. In fact, several reports have conceptually connected oxidative burst, membrane lipid peroxidation, and necrosis (Deighton

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et al. 1999, Rustérucci et al. 1999). In spite of this, and regardless of the apparently obligatory nature of the relationship between ROS and lipid peroxidation of membranes, it is clear that excess of the former compounds may end in cell death (Rustérucci et al. 1999, Overmyer et al. 2003).

Antioxidant mechanisms are present in all living organisms, and in plants they have been studied with particular emphasis in host–pathogen interactions (Hammond-Kosack and Jones 1996, Wojtaszek 1997, Bolwell et al. 2002) and to explain how plants deal with extreme environmental conditions (Schraudner et al. 1998, Vranová et al. 2002). In contrast, seaweeds have been poorly studied, though it is well known that species living in the intertidal rocky shores around the world must tolerate extreme and constantly fluctuating environmental conditions resulting from the tidal cycles. In this context, for example, several reports have confirmed that desiccation causes oxidative stress in species such as *Stictosiphonia arbuscula* (Burritt et al. 2002), *Fucus* spp. (Collén and Davison 1999a), *Mastocarpus stellatus*, and *Chondrus crispus* (Collén and Davison 1999b).

Superimposed on the natural abiotic oscillations associated with the tidal cycles, seaweeds are also exposed to other various sources of stress, particularly those resulting from human industrial, urban, and agricultural activities and their associated wastes discharges. Among these is copper mining, whose wastes have reportedly caused severe and negative effects on the coasts of England (Bryan and Langston 1992), Canada (Mardsen and DeWreede 2000, Grout and Levings 2001), Australia (Stauber et al. 2001), and Chile (Correa et al. 1999, Lancellotti and Stotz 2004). Although copper is a micronutrient for plants and animals and it naturally occurs in coastal seawater at levels at or below $1\ \mu g \cdot L^{-1}$ (Sunda 1989, Batley 1995, Apte and Day 1998), at higher concentrations it becomes toxic. The phenomenon of toxicity in algae is strongly influenced by the speciation of the metal (Gledhill et al. 1997), and within the cell it likely operates through the Haber-Weiss reaction, characterized by a heavy metal–catalyzed production of hydroxyl radicals from hydrogen peroxide (Baker and Orlandi 1995).

In northern Chile, mine wastes originating at a copper mine pit are disposed directly into the sea. The rocky intertidal zone along the impacted coast shows a severe reduction in species richness, and the macroalgal assemblage is reduced to the opportunistic algae *Ulva compressa* (Chlorophyta), formerly *Enteromorpha compressa* (Hayden et al. 2003) and *Scytosiphon lomentaria* (Phaeophyceae) (Correa et al. 1996, 1999, Medina et al. 2005). This negative effect on the biota has been reportedly recognized as the result of the persistently high levels of copper in the water, by far the most important metal brought into the system by the mine wastes (Lee et al. 2001, 2002, Medina et al. 2005). Our previous studies demonstrated that juvenile and adult plants of *U. compressa* are highly tolerant to copper (Correa et al. 1996) and that adults have powerful antioxidant defenses to buffer the oxidative stress when growing in copper-enriched environments (Ratkevicius et al. 2003). For *S. lomentaria*, on the other hand, there is no information regarding its level of tolerance to copper or the eventual mechanisms that allow this species to thrive and coexist with *U. compressa*.

In addition to the above, and from an evolutionary point of view, a persistent stress may act as a selective force and, at least theoretically, could lead to the development of ecotypes genetically adapted to the new environmental conditions. In northern Chile, the copper mine wastes have been continuously discharged for more than 60 years, long enough to expect that species with short life cycles, such as *S. lomentaria* and *U. compressa*, could have evolved into populations of individuals genetically adapted to chronically high copper levels. In fact, based on laboratory assays and using developmental parameters as end points, we tested this hypothesis (Correa et al. 1996) and found that adult individuals of *U. compressa* from a copper-enriched environment tolerated higher levels of metal than algae from pristine areas. However, juveniles of *U. compressa* responded identically to increased levels of copper, regardless the origin of their parents. Thus, at least for *U. compressa*, tolerance to high levels of copper in the water does not appear to be an inherited character (Correa et al. 1996).

In this study we address the hypothesis that *S. lomentaria* flourishes in a copper-enriched environment due to its ability to efficiently buffer the oxidative stress resulting from exposure to high levels of copper. Furthermore, and using reciprocal transplants, we tested the hypothesis that individuals from the copper-enriched area were genetically adapted to high metal levels. In this case, we expected that plants from a pristine area would not have the antioxidant capacity to overcome the level of metal stress existing at the mine-impacted area.

MATERIALS AND METHODS

Study sites. Caleta Palito (26°15′ S, 70°40′ W) and Caleta Zenteno (26°51′ S, 70°48′ W) were selected as the study sites because of their contrasting history of mining-related impact. Caleta Palito (impacted site), located in northern Chile, has received liquid-waste discharges from the copper mine in El Salvador for more than 60 years, which has resulted in the persistent enrichment of the seawater with copper (current levels of 17–29 $\mu g \cdot L^{-1}$) (Correa et al. 2000, Ratkevicius et al. 2003, Medina et al. 2005, Stauber et al. DOI 10.1016/j.marpolbul.2005.05.008) and mine sediments along approximately 30 km of coastline. Caleta Zenteno (control site) is a deserted cove, 70 km south of Caleta Palito, used sporadically by small groups of nomadic fishermen. Cachagua (32°34′ S, 71°28′ W), a small village located in central Chile (800 km south from Caleta Palito) and far away from any effect of copper mining, was used as a collecting site for *Scytosiphon lomentaria*. Caleta Zenteno, on the other hand, due to its proximity to the mine-impacted area, was chosen as the control site for the transplant experiments. Both Cachagua and Caleta Zenteno displayed normal levels of copper in the water, ranging from <1 to $5\ \mu g \cdot L^{-1}$ accompanied by

normal biological diversity occurring at the rocky intertidal zone (Ratkevicius et al. 2003, Medina et al. 2005, Stauber et al. in press).

Sampling of seawater and algae for copper analyses. Water samples were collected along 100 m of coastline in each site using three individual acid-washed plastic bottles per site. Each bottle was filled with water collected at three different locations within the selected site, mixed, and stored at 4° C. To determine copper in the tissues of *S. lomentaria*, individual thalli were collected over 200 m of intertidal platforms at the selected sites, pooled, divided into three replicates of 10–20 individuals each, blotted dry, placed in acid-washed labeled plastic bags, and transported to the laboratory in a cooler at 4° C.

Analyses of copper in seawater and algae. Seawater samples were filtered through 0.45-μm pore size membrane filters (Millipore, Bedford, MA, USA) and acidified to pH 2 using concentrated HCl (Suprapur, Merck, Darmstadt, Germany). They were then analyzed for copper by anodic redissolution potentiometry using a Tracelab Striping Potentiometer (Radiometer, Copenhagen, Sweden). Algal tissues were analyzed for copper by atomic absorption spectrophotometry in a spectrophotometer (model 55, Varian, San Francisco, CA, USA). Algal tissue was rinsed with deionized water and dried to constant weight in a ventilated oven at 60° C. A total of 0.25 g of dried tissue was digested with 6 mL of HNO_3 and 1 mL of H_2O_2 in a microwave oven (Milestone, Sorisole, Italy) for 20 min.Certified copper standards, CASS-2 for water and DOLT-1 for tissue (National Research Council, Montreal, Canada) were run simultaneously to the water and tissue samples.

EM analyses. Thalli of *S. lomentaria* from Caleta Zenteno and Caleta Palito were fixed in SFC culture medium containing 3% glutaraldehyde, 1% p-formaldehyde, and 0.1% caffeine for 3 days at 5° C, according to Correa and McLachlan (1991). Samples were postfixed in 2% OsO_4–1% potassium hexacyanoferrate − 0.05 M sodium cacodylate buffer, pH 7.8, for 2 h at 5° C, dehydrated using ethanol series (10 to 100%), and embedded in Spurr's resin for 3 days. Sections for EM were stained with uranyl acetate-lead citrate (Reynolds 1963). Samples were analyzed using a Tecnai 12 electron microscope (Phillips, Eindhoven, The Netherlands) operated at 60 kV.

Algal samples for the analysis of the antioxidant system. *Scytosiphon lomentaria* is a small-size species, and therefore individual thalli do not provide sufficient biomass to analyze antioxidant enzymes and compounds. Thus, individual thalli were collected over 200 m of intertidal platforms at the selected sites, pooled, divided into three replicates of 30–40 individuals each, blotted dry, and frozen on site in liquid nitrogen.

Detection of ROS. Reactive oxygen species were determined by a modification of the procedure described by Malanga and Puntarulo (1995). Individuals of *S. lomentaria* (1–2 g fresh weight) were collected in Cachagua and Caleta Palito and immediately incubated for 1 h at 15° C in 100 mL of 5 μM 2,4-dichlorofluoresceine diacetate (Calbiochem, San Diego, CA, USA) dissolved in 0.2 μm filtered seawater. After incubation, the tissue was rinsed in seawater, blotted dry, weighed, and frozen in liquid nitrogen. The tissue was then ground in liquid nitrogen, suspended in 5 mL of 40 mM Tris-HCl buffer, pH 7.0, and centrifuged at 20,600 *g* for 15 min. Fluorescence was determined in an LS-5 spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm. Fluorescence values were obtained using a standard curve prepared with 0–500 nM 2,4-dichlorofluoresceine (DCF; Sigma, St. Louis, MO, USA).

Detection of lipoperoxides. Lipoperoxides were determined according to Heath and Packer (1968). Algal tissue (1–2 g dry weight) from Cachagua and Caleta Palito was frozen in liquid nitrogen in a mortar and homogenized with a pestle. A total of 5 mL of 0.1% of trichloroacetic acid was added during the homogenization. The homogenate was transferred to a 30-mL glass tube and centrifuged at 7400 *g* for 20 min. Lipoperoxides were detected by addition of 100 μL of the clear homogenate to a reaction mixture containing 0.5% thiobarbituric acid (solubilized in 20% trichloroacetic acid) in a final volume of 1 mL. The reaction mixture was incubated in boiling water for 30 min, and the absorbance was measured at 512 nm.To determine the amount of lipoperoxides, the extinction coefficient of the synthesized adduct was used ($\varepsilon = 155\ mM^{-1} \cdot cm^{-1}$).

Preparation of protein extracts. Algal tissue (20 g fresh weight) was frozen in liquid nitrogen and homogenized in a prechilled mortar using a pestle. A total of 60 mL of 0.1 M phosphate buffer pH 7.0, containing 5 mM 2-mercaptoethanol, was added during the homogenization. The homogenate was allowed to thaw at room temperature, filtered through Miracloth paper (Calbiochem), and centrifuged at 7400 *g* for 15 min at 4° C. Proteins were precipitated by addition of 0.7 g of ammonium sulfate per mL of extract during 2.5 h at 4° C. The protein pellet was dissolved in 2 mL of 0.1 M phosphate buffer pH 7.0, containing 2 mM 2-mercaptoethanol and 20% glycerol. Protein concentration was determined according to Smith et al. (1985) using BSA as standard. Final protein extracts (2–4 $mg \cdot mL^{-1}$) were stored at − 80° C.

Detection of antioxidant enzyme activities. The ascorbate peroxidase (AP) activity was determined according to Chen and Asada (1989). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, 800 μM ascorbate (ASC), and 16 mM H_2O_2. After the addition of ASC, its consumption was determined at 290 nm for 1 min, and the activity was calculated using the extinction coefficient of ASC ($\varepsilon = 2.8\ mM^{-1} \cdot cm^{-1}$). The catalase (CAT) activity was determined according to Aebi (1984). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, and 10 mM H_2O_2. After the addition of H_2O_2, its consumption was determined at 240 nm for 1 min, and the activity was calculated using the extinction coefficient of H_2O_2 ($\varepsilon = 39.4\ mM^{-1} \cdot cm^{-1}$). The monodehydroascorbate reductase (MDHAR) activity was determined according to Hossain et al. (1984). The reaction mixture contained 0.1 M phosphate buffer pH 7.0, 4 mM ASC, 0.1 mM NADH, and 0.5 U of ASC oxidase (Sigma). After the addition of NADH, its consumption was determined at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$). The dehydroascorbate reductase (DHAR) activity was determined according to Miyake and Asada (1992). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, 2 mM reduced glutathione (GSH), and 300 μM dehydroascorbate (DHA). After the addition of DHA, its reduction to ASC was monitored at 265 nm for 1 min, and the activity was calculated using the extinction coefficient of ASC ($\varepsilon = 2.8\ mM^{-1} \cdot cm^{-1}$). Glutathione reductase (GR) activity was determined according to Schaedle and Bassham (1977). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, 0.5 mM oxidized glutathione (GSSG), and 90 μM NADPH. After the addition of NADPH, its oxidation was monitored at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of NADPH ($\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$). The glutathione peroxidase (GP) activity was determined according to Ursini et al. (1985). The reaction mixture contained to 0.1 M phosphate buffer, pH 7.0, 200 μM GSH, 8 mM H_2O_2, 90 μM NADPH, and 1 U of GR (Sigma). After the addition of NADPH, its oxidation was monitored at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of NADPH ($\varepsilon = 6.2\ mM^{-1} \cdot cm^{-1}$).

Detection of water-soluble antioxidant compounds. Phenolic compounds were determined using 0.2 g of fresh tissue, frozen in liquid nitrogen and homogenized in a prechilled

mortar using a pestle. A total of 2 mL of 0.1 M phosphate buffer, pH 7.0, was added during the homogenization. The homogenate was added to a 2-mL plastic tube and centrifuged in an Eppendorf microcentrifuge at 12,800 *g* for 10 min. Aliquots of 100 µL were added to a reaction mixture containing 3% of sodium carbonate and 0.3 M Folin-Ciocalteau reagent in a final volume of 1 mL. The reaction mixture was incubated for 2 h at room temperature, and the absorbance was determined at 765 nm. Total phenolic compounds were expressed as nanoequivalents of gallic acid using a calibration curve prepared with 10 to 50 nmol of gallic acid.

The GSH and GSSG levels were determined using 0.5 g of dry tissue, ground in liquid nitrogen and homogenized in a mortar using a pestle. A total of 5 mL of 5% (w/v) sulfosalicylic acid was added during the homogenization. The homogenate was transferred to a 30-mL glass tube and centrifuged at 7400 *g* for 15 min. The clear homogenate was neutralized with 1.5 volumes of 500 mM phosphate buffer, pH 7.5. Total glutathione (GSH + GSSG) was detected by addition of 100 µL of the neutralized homogenate to a reaction mixture containing 100 mM phosphate buffer, pH 7.0, 0.15 mM NADPH, 60 µM 5,5′-dithio-bis-(2-nitrobenzoic acid), and 0.66 U of GR (Sigma) in a final volume of 1 mL. The reaction mixture was incubated for 1 h at 37° C, and the absorbance was determined at 412 nm. GSSG was detected following the same procedure described above except that 100 µL of the neutralized homogenate was previously incubated with 20 µL of 1 M 2-vynilpyridine for 1 h at room temperature. The calibration curve was prepared using 2 to 40 nmol of GSH in the same reaction mixture.

The ASC and DHA were determined using 0.5 g of dry tissue, ground in liquid nitrogen and homogenized in a mortar with a pestle. A total of 5 mL of 2.5 M perchloric acid was added during the homogenization. The homogenate was transferred to a 30-mL glass tube and centrifuged at 7400 *g* for 15 min. ASC was detected by addition of 100 µL of the clear homogenate to a reaction mixture containing 2% (w/v) trichloroacetic acid, 8.8% orthophosphoric acid, 0.01% α,α′-dipyridyl, and 10 mM ferric chloride in a final volume of 1 mL. The reaction mixture was incubated for 1 h at 40° C, and the absorbance was determined at 525 nm.Total ascorbate (ASC + DHA) was detected following the same procedure described above except that 100 µL of the clear homogenate was previously incubated with 5 µL of 100 mM dithiothreitol for 1 h at room temperature. Dithiothreitol was subsequently inactivated by addition of 5 µL of 5% (w/v) *N*-ethylmaleimide. The calibration curve was prepared using 10 to 300 nmol of ASC in the same reaction mixture.

Transplant experiments. To assess the potential development of a copper-tolerant ecotype of *S. lomentaria* at the copper-enriched locality, reciprocal and simultaneous transplants were done between Caleta Palito and Caleta Zenteno. Approximately 1700 thalli of *S. lomentaria* collected in Caleta Palito were pooled and randomly assigned to transplant units. Each transplant unit consisted of a bag made of plastic net, with mesh small enough to prevent the passage of the thalli. A total of 40 individual thalli was assigned to each of the 48 transplant units from Caleta Palito. Half of these units were transferred to Caleta Zenteno and the others remained in Caleta Palito as controls for the transplants. A similar approach was followed in Caleta Zenteno, although in this case local *S. lomentaria* individuals were supplemented with plants brought from Cachagua, which were acclimated for 24 h before the beginning of the experiment. Transport of the transplant units from Caleta Palito to Caleta Zenteno was done simultaneously with that from Caleta Zenteno to Caleta Palito. Transplant units were transported in coolers with water from their respective sites. Once transplant units arrived to the receiving site, they were fastened to a rope anchored to the rocky bottom in a way that samples could be collected at predetermined times, regardless of tide height. Transplants were set up at the same time at Caleta Palito and Caleta Zenteno, after which triplicate samples were simultaneously collected at 0, 3, 6, 18, 24, 48, and 96 h. Samples were also collected at the moment of removing the plants from the rocks and after transplantation (but before starting the experiment) to assess the preexperimental status of the algae. Material sampled during this experiment was frozen on site in liquid nitrogen and transported to the laboratory for analysis.

Statistical analysis. Detections of ROS, lipoperoxides, and water-soluble antioxidant compounds were done in triplicate. Activity of each antioxidant enzyme was measured in three different extracts prepared independently. The significance of the observed differences between algae exposed to the environment of Caleta Palito and Caleta Zenteno was assessed using the Student's *t*-test (Sokal and Rohlf 1981).

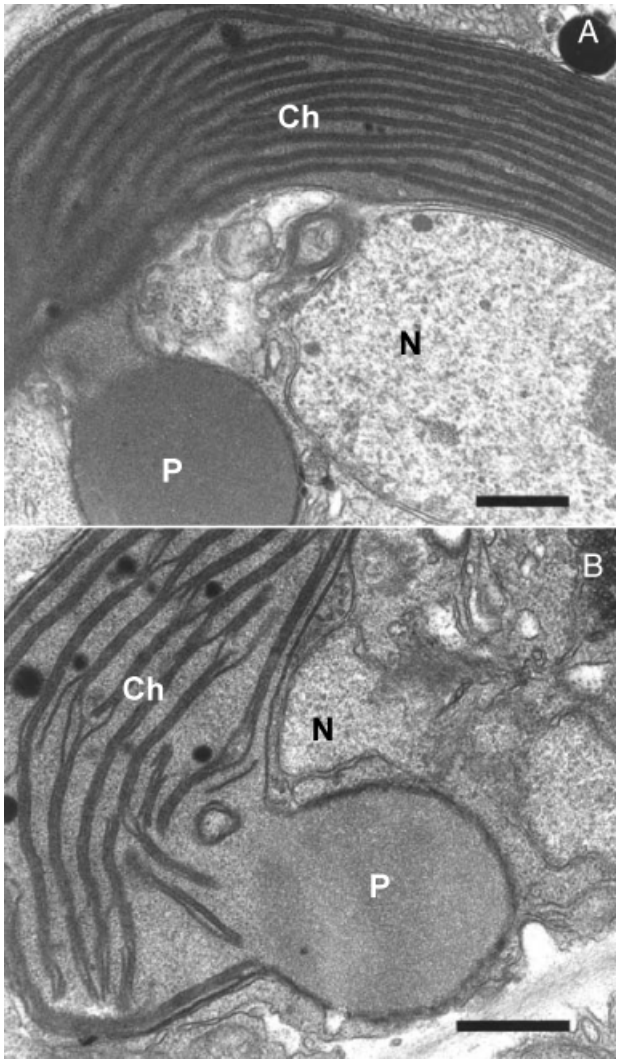

FIG. 1. Ultrastructure of *Scytosiphon lomentaria* from the control site (A) and from the copper-enriched site (B). No differences are apparent, and chloroplast (Ch), pyrenoid (P), and nucleus (N) display a normal morphology. Scale bar, 500 nm.

RESULTS

Copper levels in seawater and in Scytosiphon lomentaria. The coastal water around Caleta Palito, the area under the effect of the mine wastes, showed a clearly higher level of total dissolved copper ($10.7 \pm 1.1\ \mu g \cdot L^{-1}$, $n = 3$) than the level recorded in water samples from the control site ($1.3 \pm 0.06\ \mu g \cdot L^{-1}$, $n = 3$). These differences in water were also reflected in the copper levels within the tissues of *S. lomentaria* because those chronically exposed to the copper-enriched water of Palito contained 20 times more copper ($151.3 \pm 40.2\ \mu g \cdot g^{-1}$ dry tissue) than those from the control site ($8.1 \pm 1.0\ \mu g \cdot g^{-1}$ dry tissue).

Ultrastructure of Scytosiphon lomentaria. The structure of *S. lomentaria* cells from the control (Fig. 1A) and impacted sites (Fig. 1B) was normal, in spite of the higher levels of copper to which the algae growing at the mine-impacted site were exposed. This is particularly clear in the chloroplast of algae from the impacted site (Fig. 1B), where the external double membrane surrounds the normal phaeophycean organization of thylakoids in stacks of three lamellae. Furthermore, structurally normal pyrenoids and nuclei were present in all cells.

Reactive oxygen species and lipoperoxide levels. The levels of the two indicators of oxidative stress selected in this study appeared to be significantly increased in *S. lomentaria* from Caleta Palito (Fig. 2, A and B). Reactive oxygen species were 24 times higher in plants from the copper-enriched area, with 187.7 nmol $DCF \cdot g^{-1}$ fresh tissue, than in those from the control site, with 7.8 nmol $DCF \cdot g^{-1}$ fresh tissue. In the case of lipoperoxides, on the other hand, 72.5 nmol $\cdot g^{-1}$ dry tissue were recorded in algae from the copper-enriched area, whereas only 20.2 nmol $\cdot g^{-1}$ dry tissue were measured in plants from the control site.

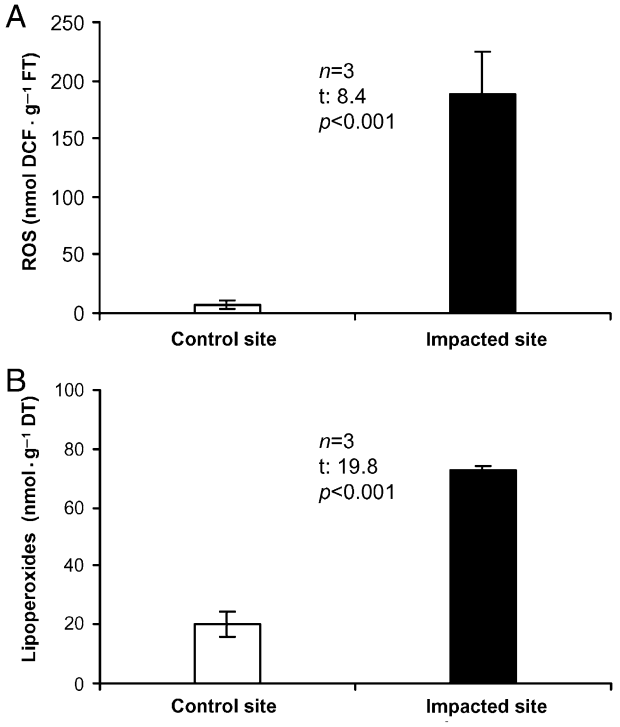

FIG. 2. Levels of ROS and lipoperoxides in *Scytosiphon lomentaria* from the control site (white bars) and the copper-impacted site (black bars). ROS levels (A) are expressed per gram of fresh tissue (FT) and lipoperoxide levels (B) per gram of dry tissue (DT). Bars represent mean values of three independent replicates ± 1 SD.

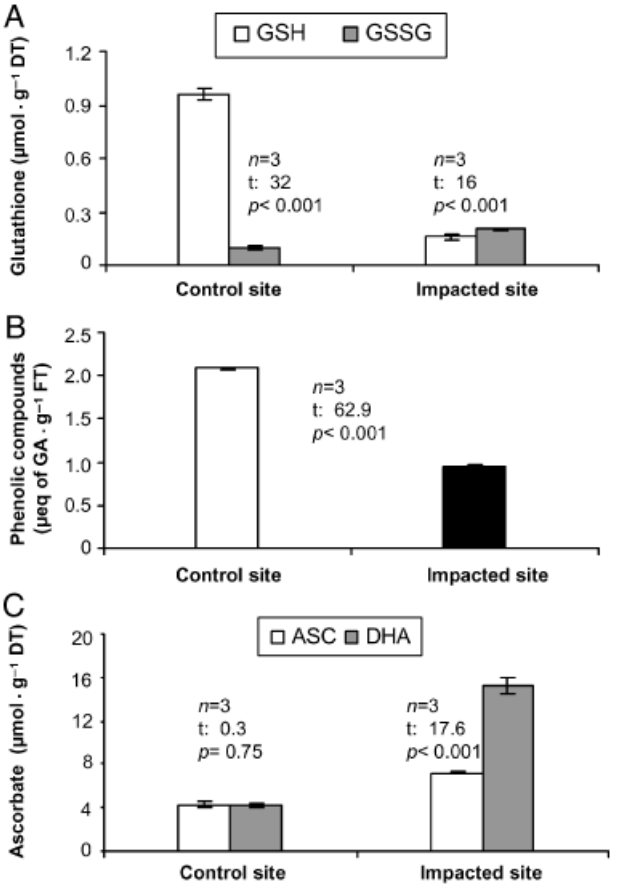

FIG. 3. Levels of antioxidant compounds in *Scytosiphon lomentaria* from the control site and the copper-impacted site. Levels of GSH and GSSG (A), ASC and DHA (C) are expressed per gram of dry tissue (DT) and phenolic compound levels (B) per gram of fresh tissue (FT). Bars represent mean values of three independent replicates ±1 SD.

Water-soluble antioxidant compounds. In general terms, and with the exception of ASC, water-soluble antioxidant compounds were lower in *S. lomentaria* from the copper-enriched site (Fig. 3, A–C). In the case of glutathione, the reduction of the total pool is due to the significant decrease ($t = 40.5$, $P < 0.001$) in GSH to less than 20% of the values recorded in algae from the control site, from 0.96 to 0.16 µmol $\cdot g^{-1}$ dry tissue (Fig. 3A). On the other hand, GSSG was more than twice as high in algae from the copper-enriched area than in those from the control site ($t = 3$; $P = 0.039$), with values of 0.2 µmol $\cdot g^{-1}$ dry tissue and 0.096 µmol $\cdot g^{-1}$ dry tissue, respectively (Fig. 3A). Similarly, the levels of water-soluble phenolic

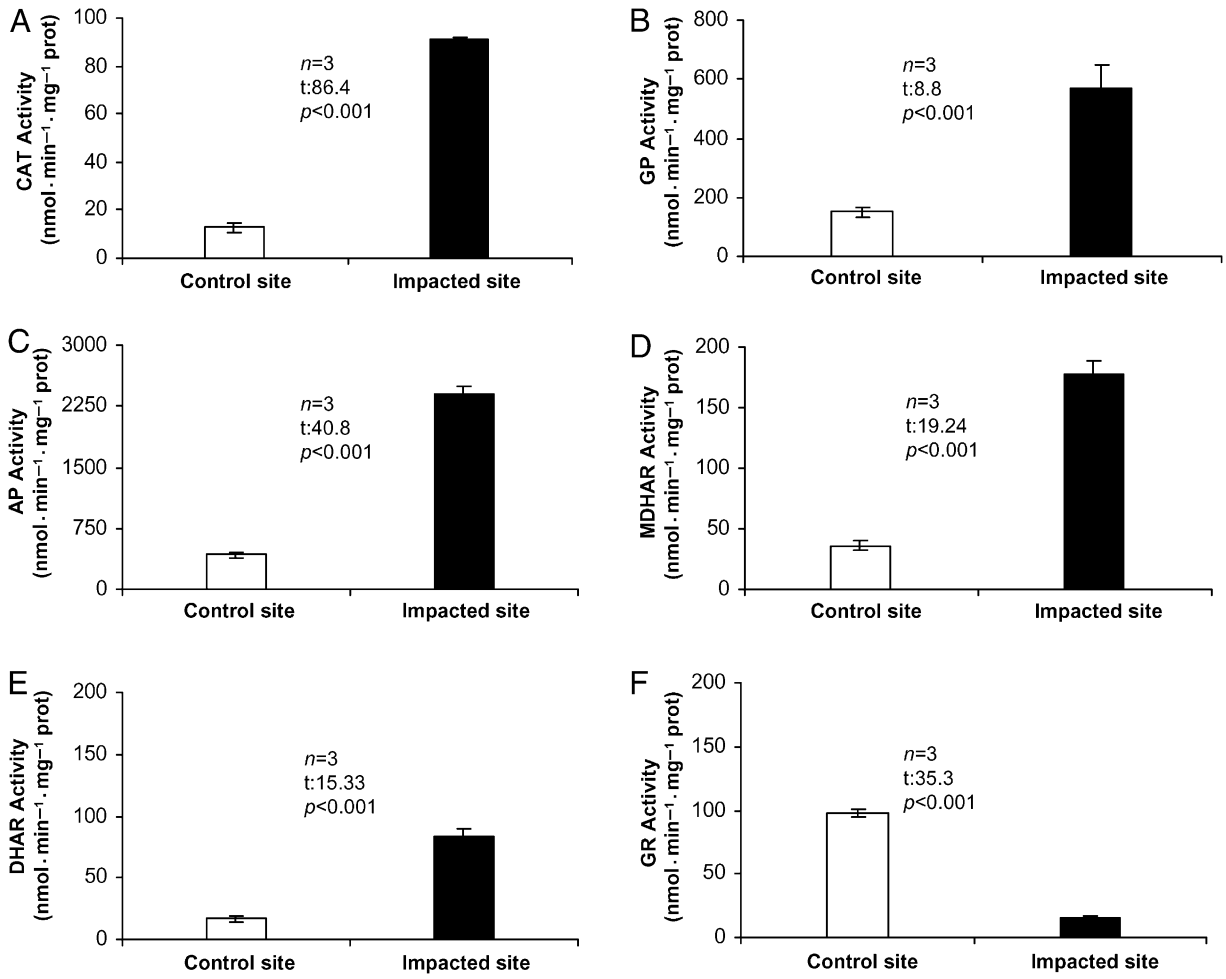

FIG. 4. Activities of the antioxidant enzymes (A) CAT, (B) GP, (C) AP, (D) MDHAR, (E) DHAR, and (F) GR in *Scytosiphon lomentaria* from the control site (white bars) and the copper-impacted site (black bars). The activities of (A) CAT, (B) GP, (C) AP, (D) MDHAR, (E) DHAR, and (F) GR. Bars represent mean values of three independent replicates ±1 SD.

compounds decreased significantly ($t = 63$, $P < 0.001$) from 2.1 μequivalents of gallic acid · g^{-1} fresh tissue in plants from the control site to 0.94 μequivalents of gallic acid · g^{-1} fresh tissue in algae from the copper-enriched site (Fig. 3B). In contrast, the main effect of copper enrichment on the pool of ASC (ASC + DHA) was the significant accumulation of DHA ($t = 23.4$, $P < 0.001$), from 4.2 μmol · g^{-1} dry tissue in control plants to 15.2 μmol · g^{-1} dry tissue in those from the copper-enriched area (Fig. 3C). Levels of ASC were also significantly higher ($t = 17.3$, $P < 0.001$) in plants from the copper-enriched area, with 7.2 μmol · g^{-1} dry tissue, than those recorded in control plants, where mean values of 4.3 μmol · g^{-1} dry tissue were recorded (Fig. 3C).

Antioxidant enzymes activities. With the exception of GR, all antioxidant enzyme activities appeared to be increased in *S. lomentaria* from the copper-enriched site (Fig. 4). The CAT activity displayed the highest increase, 7-fold among the antioxidant enzymes tested, with values of 13 nmol · min^{-1} · mg^{-1} protein in the controls and 90.7 nmol · min^{-1} · mg^{-1} protein in the plants from the copper-enriched site (Fig. 4A). Similarly, GP activity was 4-fold higher, with a mean value of 149 nmol · min^{-1} · mg^{-1} in control algae compared with 568 nmol · min^{-1} · mg^{-1} protein in algae from the copper-enriched site (Fig. 4B). The enzymes of the Halliwell-Asada cycle, AP, MDHAR, and DHAR activities, were 6-fold, 5-fold, and 5-fold, respectively, in algae from the copper-enriched site than in algae from the control site (Fig. 4, C–E). In contrast, GR activity (Fig. 4F) was strongly inhibited in algae from the copper-enriched site, as revealed by the significant decrease ($t = 32.6$, $P < 0.001$) from 97.8 nmol · min^{-1} · mg^{-1} in plants from the control site to 15.2 nmol · min^{-1} · mg^{-1} protein in those from the copper-enriched site.

Plasticity of the antioxidant responses in cross-transplanted Scytosiphon lomentaria. Lipoperoxide and antioxidant compound levels and antioxidant enzyme activities did not change in any control for the transplant manipulation during the 96 h of the experiment (Figs. 5 and 6). In contrast, lipoperoxide levels increased in algae transplanted from the

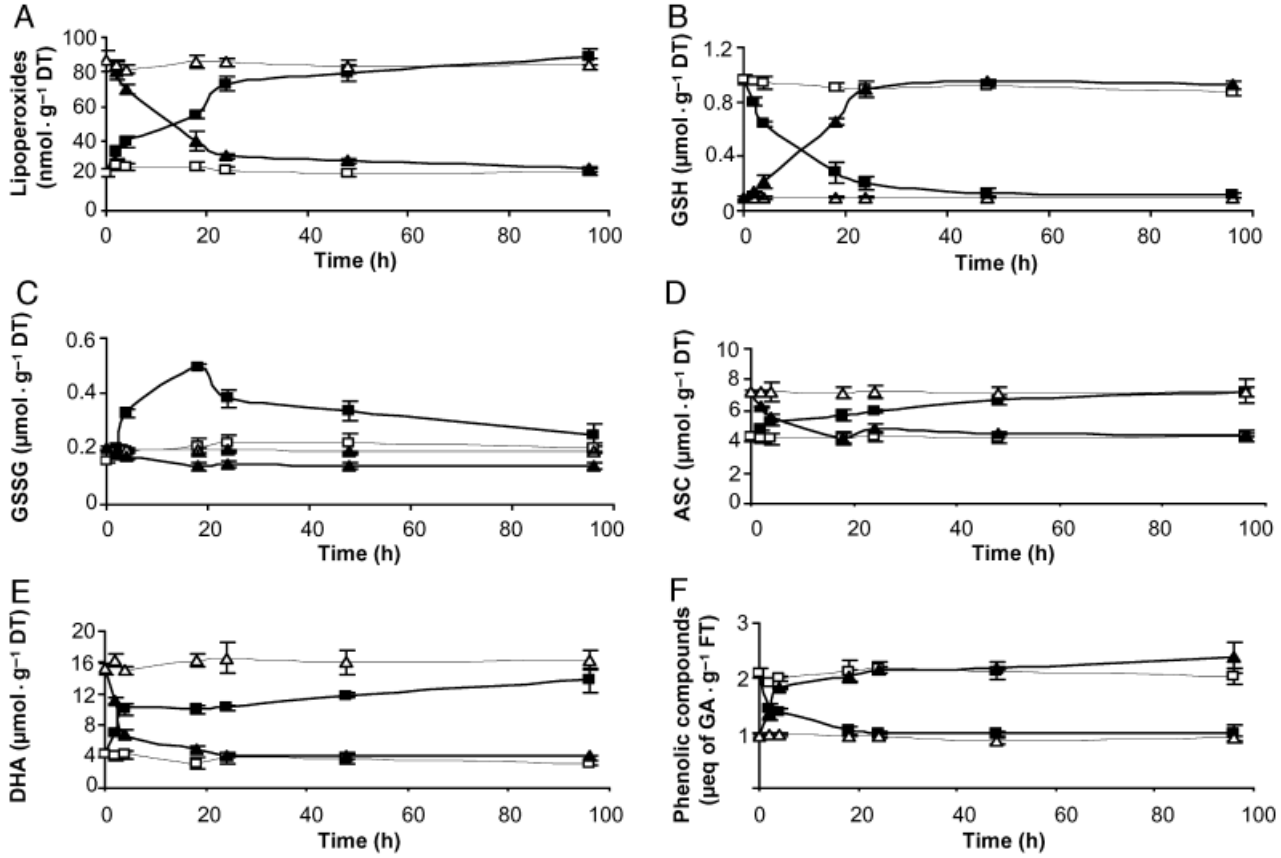

FIG. 5. Temporal changes in levels of lipoperoxides, and antioxidant compounds in cross-transplanted *Scytosiphon lomentaria*. Levels of (A) lipoperoxides, (B) GSH, (C) GSSG, (D) ASC, (E) DHA, and (F) phenolic compounds were determined in transplants within the control site (□), within the copper-impacted site (Δ), from the control site to the copper-impacted site (■), and from the copper-impacted site to the control site (▲). Levels of lipoperoxides GSH, GSSG, ASC, and DHA are expressed per gram of dry tissue (DT) and levels of phenolic compounds per gram of fresh tissue (FT). Each point represents mean values of three independent replicates ± 1 SD.

control to the copper-enriched site and decreased in those algae transplanted from the copper-enriched area to the control site (Fig. 5A). The levels of lipoperoxide in both cases were almost completely reversed in 48 h, and after 96 h the mean value of lipoperoxides in the transplants did not differ from their respective local controls (Fig. 5A). The same trend was found in the case of GSH, GSSG, ASC, DHA, and phenolic compounds (Fig. 5, B–F). The accumulation or depletion of GSSG and DHA was completed in 96 h. In addition to the above, the kinetic of the responses to oxidative stress by the antioxidant compounds revealed that the depletion/replacement rate varied according to the compound involved. Thus, whereas the pool of GSH in algae transplanted from the control site to the copper-enriched site was depleted in 48 h, it took only 24 h for *S. lomentaria* transplanted from the copper-enriched site to the control site to recover the normal level of this compound (Fig. 5B).

In the case of the water-soluble phenolic compounds, both depletion and replacement of the pool occurred 18 h after the beginning of the experiment (Fig. 5F). With ASC, where the basal level in algae from the control site was lower than in those from the mine-impacted site, the recovery of the normal levels (i.e. the depletion of the pool in copper-enriched plants) took place 18 h after the transplantation, whereas it took 48 h for the control algae to reach the ASC levels of those normally growing at the copper-enriched area (Fig. 5D). It is also important to mention that the responses of GSSG and DHA in transplanted individuals (Fig. 5, C and E) appeared delayed in comparison with those recorded for their respective reduced precursors, GSH and ASC. In fact, individuals transplanted from the copper-enriched to the control site never reached the level of GSSG recorded in algae normally growing at the control site (Fig. 5C, $t = 2.7$, $P = 0.047$) within the time frame of the experiment (i.e. 96 h).

The activities of CAT, GP, AP, MDHAR, DHAR, and GR showed the same pattern of reversibility displayed by the antioxidant compounds, and the process was completed in 96 h in each case (Fig. 6). In summary, lipoperoxides and antioxidant compound levels and antioxidant enzyme activities showed a complete reversibility in algae transplanted from a nonimpacted site to a copper-enriched site, and vice versa.

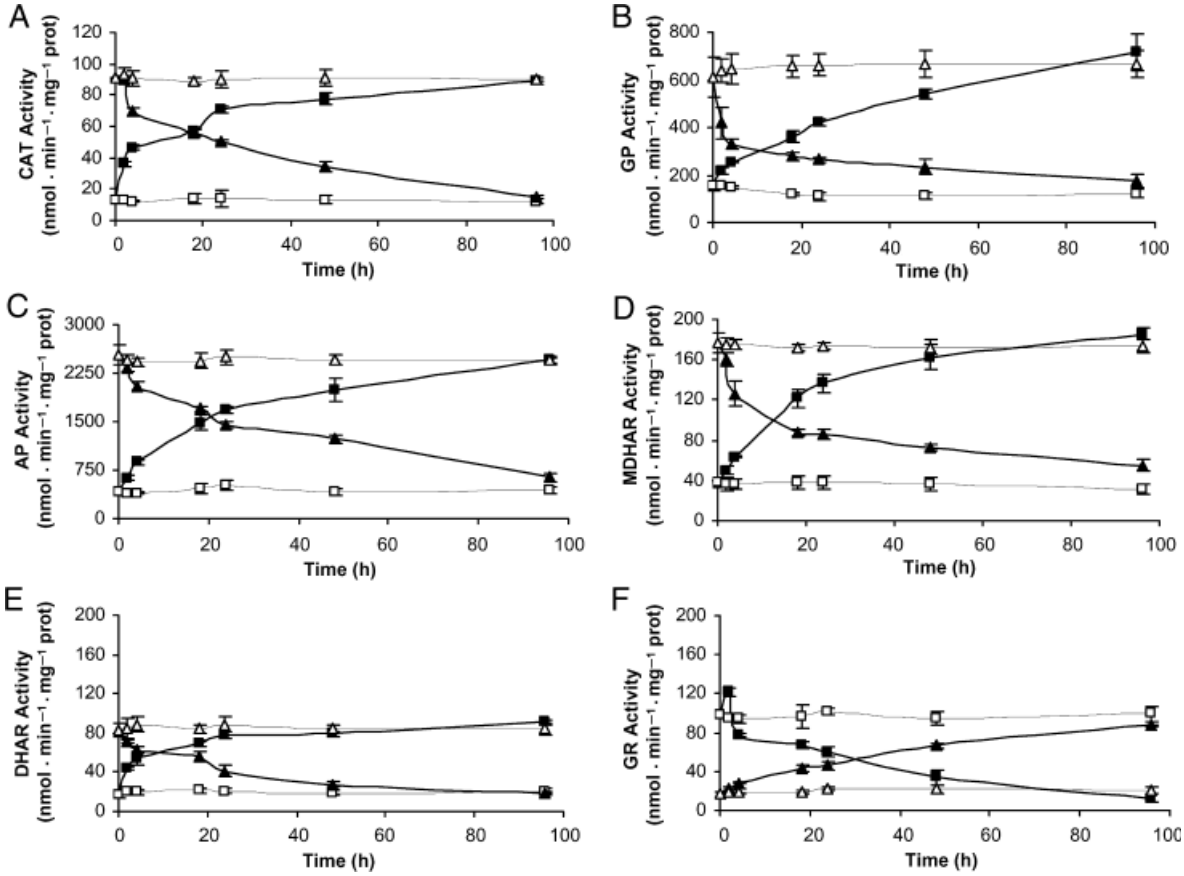

FIG. 6. Temporal changes in antioxidant enzymes activities in cross-transplanted *Scytosiphon lomentaria*. The activities of (A) CAT, (B) GP, (C) AP, (D) MDHAR, (E) DHAR, and (F) GR were determined in transplants within the control site (□), within the copper-impacted site (△), from the control site to the copper-impacted site (■), and from the copper-impacted site to the control site (▲). Each point represents mean values of three independent replicates ±1 SD.

DISCUSSION

Our results demonstrated that the coastal copper-enriched environment around the discharges of the copper mine from El Salvador is responsible for an imbalance in the redox status of *S. lomentaria*, which was revealed by an increase in ROS and lipoperoxide levels and changes in the antioxidant system (i.e. antioxidant compounds and enzymes) of the algae. Indeed, copper remains, by far, the main metal in the mine-impacted site under study, reaching a concentration of an order of magnitude higher than in control sites (Correa et al. 1996, 1999, Lee et al. 2001, 2002, Ratkevicius et al. 2003, this work). The higher concentration of copper in the water resulted in accumulation of the metal in the tissues of *S. lomentaria* at concentrations almost 20 times higher than in algae from the control site. The accumulation in *S. lomentaria* was not completely unexpected, as similar results have been reported for *U. compressa*, the only species that coexists in large densities with *S. lomentaria* at the copper-enriched site (Ratkevicius et al. 2003) and for a variety of algae inhabiting metal-enriched coastal environments around the world (Foster 1976, Strömgren 1980, Luoma et al. 1982, Ho 1984, 1990, Forsberg et al. 1988, Say et al. 1990, Mardsen and DeWreede 2000). The high levels of copper in the tissues of *S. lomentaria* naturally growing at the impacted site was not reflected in changes at the organellar level, as reported for other seaweeds such as *Lessonia nigrescens* (Leonardi and Vásquez 1999) and *Enteromorpha flexuosa* (Andrade et al. 2004). This apparent absence of effects on the structure of the cells may well be the result of the attenuation of copper toxicity due to the scavenging activity of the antioxidant system of *S. lomentaria*, as discussed below.

The accumulation of high levels of copper in the tissues of *S. lomentaria* from the copper-enriched area was associated with a significant increase in ROS and lipoperoxide levels. Information regarding the relationship between copper enrichment and increased ROS levels in seaweeds is not available. However, even if ROS are considered normal byproducts of cell metabolism (Asada 1999, Dat et al. 2003), their net increase is an unequivocal indication of an oxidative stress condition (Baker and Orlandi 1995, Livingstone 2001, Mittler 2002). Thus, the clearly higher level of ROS in algae from the area under the influence of the

mine wastes strongly suggests that *S. lomentaria* from Palito was under a condition of copper-mediated oxidative stress.

There is a general agreement in that a condition of oxidative stress results in the presence of abnormally high levels of oxidized macromolecules, including lipoperoxides (Rustérucci et al. 1999, Imbusch and Mueller 2000). Our results are consistent with this suggestion, as the levels of lipoperoxides in algae from the copper-enriched area were almost four times higher than in algae from the control sites. However, the increase in lipoperoxides in *S. lomentaria* from the copper-enriched area requires special consideration. When standardized on a fresh weight basis, levels of ROS and lipoperoxides in algae from the control site are in a 1:1 ratio. In contrast, the levels of ROS in algae from the impacted area increased 24 times, whereas their lipoperoxide levels increased only 4 times. The relatively low level of lipoperoxides compared with ROS in copper-stressed algae suggests that either production of lipoperoxides is attenuated by the antioxidant
system or that their degradation is increased, or both. A commonly used pathway for eliminating the end products from lipoperoxide degradation is their condensation with glutathione, a reaction catalyzed by glutathione-S-transferase (Deighton et al. 1999, Thoma et al. 2003). In this context, we detected a 3-fold increase in the activity of glutathione-S-transferase in algae from the impacted site (unpublished data), which may represent a mechanism that complements the action of the antioxidant system.

Our results also showed that the development of an oxidative stress condition in *S. lomentaria* from the copper-enriched area is associated with important changes in the antioxidant system. Concerning the water-soluble antioxidant compounds, consumption of GSH and phenolic compounds, which decreased 6- and 2-fold, respectively, accompanied by a 2-fold accumulation of ASC, were detected in algae from the copper-enriched area. These patterns of changes, as well as their magnitude, are similar to those reported for vascular plants stressed by heavy metals (Gallego et al. 1996, Gupta et al. 1999, Rao and Sresty 2000, Sgherri et al. 2003). However, in the green alga *U. compressa*, the only other seaweed where changes in water-soluble antioxidant compounds triggered by copper enrichments have been studied, the magnitude of the response is strikingly different. In thalli of *U. compressa* collected at the same site where *S. lomentaria* was obtained for this study, levels of GSH, phenolic compounds, and ASC decreased approximately 280-, 13-, and 6-fold, respectively, as compared with those levels measured in pristine environments (Ratkevicius et al. 2003). In contrast, changes in the antioxidant compounds in *S. lomentaria* are much closer to those reported for other macroalgae and vascular plants exposed to various abiotic stresses (Ederli et al. 1997, Hodges and Forney 1997, Collén and Davidson 1999a,b, Hodges et al. 2000, Burritt et al. 2002). Another clear difference became apparent when comparing DHA accumulation in *S. lomentaria* (four times higher) and *U. compressa* (35 times higher) with their respective nonstressed controls. In *S. lomentaria*, low levels of DHA seem to result from recycling this compound into ASC through DHA reductase activity that was, in fact, detected in both copper-stressed and nonstressed algae (see below). In *U. compressa*, on the other hand, DHA reductase activity was not detected, even in algae under copper stress. Therefore, it was suggested that in this species oxidative stress involved synthesis of ASC that accumulated as DHA (Ratkevicius et al. 2003). An important aspect that adds relevance to some of the changes observed in the antioxidant compounds is the potential relationship between glutathione and phytochelatins. As already suggested by Ratkevicius et al. (2003) working with *Enteromorpha compressa*, the significant consumption of GSH and GSSG could be explained by the channeling of GSH to phytochelatins, which are glutathione polymers known by their metal chelating properties (Cobbett 2000) and by their ROS scavenging activity, which is reportedly higher than GSH (Tsuji et al. 2002). Another possibility is that GSH synthesis is inhibited, although so far no evidence for a direct inhibition by copper of the enzymes involved in GSH synthesis has been reported in algae.

Simultaneously with the changes in the levels of antioxidant compounds, there was a clear change in the activity of the antioxidant enzymes in *S. lomentaria* from the copper-enriched area. Activities of all measured antioxidant enzymes, with the exception of GR, were four to seven times higher than those recorded in algae from the control sites. The pattern of these responses, as well as their magnitude, is in agreement with those reported for vascular algae exposed to copper enrichment (Weckx and Clijsters 1996, Cuypers et al. 1999, Cho and Park 2000, Rao and Sresty 2000, Dixit et al. 2001, Shützendübel and Polle 2002). There is also an agreement with the responses reported in the red macroalga *Gracilaria tenuistipitata* stressed by copper (Collén et al. 2003) and in some microalgae exposed to heavy metals (Sauser et al. 1997, Okamoto et al. 2001). As observed for the antioxidant compounds, there were striking differences between *S. lomentaria* and *U. compressa* regarding the antioxidant enzymes. Whereas in the former species copper stress triggered the activity of five antioxidant enzymes, including those of the Haliwell-Asada cycle, in *U. compressa* the entire antioxidant enzymatic response relied on the activity of AP, which increased 40–60 times over the controls (Ratkevicius et al. 2003). In spite of the above differences, the two species showed a similar inhibition of GR activity, which probably results from a direct action of copper on the enzyme, as it has been documented for GR in *Euglena gracilis* and in some terrestrial algae (Shigeoka et al. 1987, Shützendübel and Polle 2002).

The antioxidant responses of *S. lomentaria* during the reciprocal and simultaneous transalgae strongly suggest the absence of an ecotype specially adapted to

the copper levels currently present at the mine-impacted area. First, transplants of individuals from this area (i.e. a population chronically exposed to a copper-enriched environment) to the control site showed that the responses of the antioxidant system were completely reverted in a short period of time. In this case, 48 to 96 h were enough for the levels of antioxidant compounds and the activities of antioxidant enzymes to become almost identical to those displayed by algae normally growing at the control site. Second, in the transplants from the control site to the copper-enriched area, the antioxidant system of *S. lomentaria* was quickly activated to the levels of those algae naturally growing at the impacted site. Thus, the capacity of *S. lomentaria* to rapidly respond to contrasting copper environments by modifying the activity of its antioxidant system reveals an important degree of physiological plasticity, rather than genetic adaptation to a site-specific condition of copper concentration. This suggestion, however, remains to be experimentally demonstrated. For example, exposing *S. lomentaria* from the studied populations to copper levels still higher than the concentrations currently detected in Palito could take the capacity of the antioxidant system of this species to its limit and thus help to identify an eventual intrinsically higher capacity to buffer copper stress in individuals from the area under the influence of the mine wastes. The absence of ecotypes had been already reported for *U. compressa* after measuring the degree of copper tolerance expressed in the progeny of algae from a pristine locality compared with that from a population chronically exposed copper enrichment (Correa et al. 1996). The above information, however, does not agree with earlier studies, such as those done by Russel and Morris (1970) on *Ectocarpus siliculosus* and by Reed and Moffat (1983) on *Enteromorpha compressa*, where results indicated that algae from copper-enriched environments were able to tolerate higher concentrations of the metal in laboratory assays. The occurrence of ecotypes as the result of metal stress is also controversial in terrestrial plants facing metal stress, with studies supporting the phenomenon (Van Hoof et al. 2001, Shu et al. 2002) and others suggesting its absence (Boyd and Martens 1998, Ye et al. 2003). This is certainly an area that requires additional field and laboratory experimentation to understand the potential evolutionary relationship between copper enrichment and algae.

Conclusion. This study provides experimental evidence that the antioxidant system is involved in the resistance to abnormally high levels of copper in the brown alga *S. lomentaria*. Furthermore, it demonstrates that resistance to copper in this alga includes the consumption and synthesis of water-soluble antioxidant compounds and the activation of most of the antioxidant enzymes known in vascular algae. Finally, this is the first *in vivo* experimental evidence that the function of the antioxidant system involved in the phenomenon of copper resistance is plastic and reversible, which suggests that 1) resistance to chronic exposure to copper is a constitutive trait and 2) *S. lomentaria* from the population at the area chronically enriched with copper does not seem to be a copper-tolerant ecotype. Overall, the plasticity and induction capacity of the antioxidant metabolism explains, at least partially, the persistence of *S. lomentaria* in a coastal environment receiving copper mine wastes in northern Chile.

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