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Copper-induced intra-specific oxidative damage and antioxidant responses in strains of the brown alga Ectocarpus siliculosus with different pollution histories

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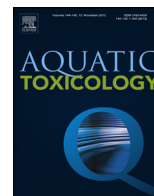Claudio A. Sáez [a,b,1], Francesca Roncarati [a,1], Alejandra Moenne [b], A. John Moody [c], Murray T. Brown [a,*]

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ABSTRACT

Inter- and intra-specific variation in metal resistance has been observed in the ecologically and economically important marine brown macroalgae (Phaeophyceae), but the mechanisms of cellular tolerance are not well elucidated. To investigate inter-population responses of brown seaweeds to copper (Cu) pollution, the extent of oxidative damage and antioxidant responses were compared in three strains of the filamentous brown seaweed *Ectocarpus siliculosus*, the model organism for the algal class Phaeophyceae that diverged from other major eukaryotic groups over a billion year ago. Strains isolated from locations with different pollution histories (*i.e.* LIA, from a pristine site in Scotland; REP and Es524 from Cu-contaminated sites in England and Chile, respectively) were exposed to total dissolved Cu concentrations (Cu_T) of up to 2.4 μM (equivalent to 128 nM Cu^{2+}) for 10 d. LIA exhibited oxidative stress, with increases in hydrogen peroxide (H_2O_2) and lipid peroxidation (measured as TBARS levels), and decreased concentrations of photosynthetic pigments. Es524 presented no apparent oxidative damage whereas in REP, TBARS increased, revealing some level of oxidative damage. Adjustments to activities of enzymes and antioxidant compounds concentrations in Es524 and REP were strain and treatment dependent. Mitigation of oxidative stress in Es524 was by increased activities of superoxide dismutases (SOD) at low Cu_T, and catalase (CAT) and ascorbate peroxidase (APX) at all Cu_T, accompanied by higher levels of antioxidants (ascorbate, glutathione, phenolics) at higher Cu_T. In REP, only APX activity increased, as did the antioxidants. For the first time evidence is presented for distinctive oxidative stress defences under excess Cu in two populations of a species of brown seaweed from environments contaminated by Cu.

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1. Introduction

Copper (Cu) is an essential micronutrient for metabolic processes in chloroxygenic organisms (Burkhead et al., 2009). However, when intracellular concentrations exceed optimal levels it can be a potent toxicant, altering physiological and biochemical functions that impair growth and development (Nagajyoti et al., 2010). In excess, metals such as Cu disturb the redox balance of cells leading to oxidative stress, which is characterized by increased production of harmful reactive oxygen species (ROS) and a shift in the

redox equilibrium to the pro-oxidative state (Connan and Stengel, 2011; Nielsen et al., 2003a). The reduction of molecular oxygen generates intermediates such as superoxide anions ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$) (Foyer and Noctor, 2011) that are potentially toxic due their oxidation of proteins, lipids, polysaccharides and nucleic acids (Nielsen et al., 2003a). However, although elevated concentrations of ROS are damaging to cellular processes, it is now known that ROS play crucial roles in modulating signal transduction pathways in photoautotrophs, including brown algae, and therefore intracellular concentrations must be tightly controlled, but not completely eliminated (Apel and Hirt, 2004; Cosse et al., 2009; Smirnoff, 2005). To re-balance the redox status and maintain ROS to within physiological concentrations, cells have an effective antioxidant defence system that comprises a battery of enzymes and antioxidant molecules. Key

enzymes include superoxide dismutases (SOD) that dismutases
$\bullet O_2^-$ to H_2O_2, and which are considered the first line of defence
against ROS (Pinto et al., 2003), catalase (CAT) that catalyses the
dismutation of H_2O_2 to oxygen and water, glutathione peroxi-
dase (GPX) that catalyses the reduction of H_2O_2 to water using
reduced glutathione (GSH) as substrate, and enzymes associated
with the Foyer–Halliwell–Asada pathway (ascorbate–glutathione
cycle) such as ascorbate peroxidase (APX) that catalyses the reduc-
tion of H_2O_2 using ascorbate (ASC) as substrate, dehydroascorbate
reductase (DHAR) that reduces dehydroascorbate (DHA) to ASC
using GSH as a substrate and glutathione reductase (GR) that
reduces oxidized glutathione (GSSG) to GSH, using NADPH as the
source of reducing power (Collen and Davison, 1999, 2001; Foyer
and Noctor, 2011). The low molecular weight antioxidants ASC and
GSH can directly reduce ROS, and serve as co-factors for reactions
catalysed by APX and GR, respectively. GSH can be oxidized to GSSG,
and ASC to dehydroascorbate (DHA) and monodehydroascorbate
(MDHA), which may unbalance redox equilibrium and affect cell
processes including spindle formation in cell division, and primary
and secondary metabolism (see Collen and Davison, 1999, 2001;
Contreras et al., 2005, 2009). Additionally, phenolic compounds can
act directly as scavengers of ROS (Connan and Stengel, 2011). While
the extensive literature affirms the view that exposure of chloroxy-
genic organisms to high concentrations of redox active metals, such
as Cu, induces oxidative damage, the specific antioxidant responses
can vary between species and amongst tissues (Collen and Davison,
1999; Pinto et al., 2003; Smirnoff, 2005).

In oceanic surface waters total dissolved Cu concentrations
(Cu_T) are maintained at pM–nM levels, whereas in coastal waters
and estuaries adjacent to areas of urbanization or receiving mine
drainage water, Cu_T concentrations can exceed 0.1 μM, values that
are 20 times higher than encountered in uncontaminated estuaries
and coastal locations (Andrade et al., 2006; Nielsen et al., 2003a).
However, it is mainly the concentrations of free ion (Cu^{2+}) as well as
kinetically labile and lipid soluble Cu complexes, and not Cu_T, that
more accurately reflects the fraction available to marine photoau-
totrophs, and directly relates to Cu toxicity (Gledhill et al., 1997;
Leal et al., 1999). In near-shore ecosystems, seaweeds (marine
macroalgae) are the dominant primary producers and the brown
seaweeds (Phaeophyceae), that are members of the Stramenopila
which diverged from green plants, fungi and animals over a bil-
lion years are ago (Cock et al., 2010), are particularly important
bio-engineers, providing shelter, food and habitat for other marine
biota (Graham et al., 2007; Mann, 1973). Our understanding of
how seaweeds are affected by excess Cu, and other metals, and
the mechanisms by which they detoxify and tolerate metals is still
comparatively poor, although the knowledge base has increased
in recent years (*e.g.* Brown et al., 2012; González et al., 2010,
2012a; Nielsen et al., 2003b; Pawlik-Skowronska et al., 2007). With
respect to biochemical responses to Cu stress, the most compre-
hensive studies, to date, have been on the green seaweed *Ulva
compressa* (Ulvophyceae) and brown seaweed *Scytosiphon lomen-
taria* (Phaeophyceae), two prominent members of the seaweed
community in Cu-enriched coastal waters (over 1000 μg L^{-1}) of
northern Chile (Lee and Correa, 2005; Ramirez et al., 2005). Chronic
exposure of *U. compressa* to Cu induced an oxidative stress con-
dition buffered mainly through the activation of the antioxidant
enzyme APX, and synthesis of ASC whereas in *S. lomentaria* protec-
tion from oxidative stress occurs *via* increased activities of mainly
CAT, GP and APX, and production of ASC (Contreras et al., 2005,
2009; González et al., 2012b; Mellado et al., 2012; Ratkevicius et al.,
2003). In both these species it was concluded that tolerance to
Cu is a constitutive trait (Contreras et al., 2005; Ratkevicius et al.,
2003). Furthermore, persistence of metal stress may lead to the
directional selection of traits that aid survivability of individuals in
polluted environments, evidence for which has been obtained for

the brown seaweeds *Fucus serratus* (Nielsen et al., 2003b) and *Ecto-
carpus siliculosus* (Russell and Morris, 1970). A recently published
proteomic analysis of two strains (Es32 and Es524) of *E. siliculosus*
from contrasting Cu-impacted locations and exposed to Cu showed
that Es524 expressed specific Cu-stress related proteins, such as
RNA helicases and a vanadium-dependent bromoperoxidase, iden-
tifying features related to Cu tolerance in this strain (Ritter et al.,
2010). In addition, it has been shown that amongst strains of *E.
siliculosus* there are high levels of morphological, physiological and
genetic variation (Dittami et al., 2011). Thus, strains of *E. siliculosus*
may display different degrees of oxidative stress and variation in
antioxidant responses to Cu stress.

To test this hypothesis, Cu-induced oxidative damage and
antioxidant responses were investigated, under laboratory condi-
tions at environmentally realistic concentrations of the metal, in
three strains of *E. siliculosus* that had been isolated from locations
with differing pollution histories and subsequently maintained in
laboratory culture. Strain LIA08-4 (LIA) is from a pristine site in
north-west Scotland (Lon Liath), strain EcREP10-11 (REP) is from a
metal-contaminated estuary in south–west England (Restronguet
Creek) that receives mine drainage water contaminated with
particularly high concentrations of Cu, zinc (Zn), arsenic (As), man-
ganese (Mn) and lead (Pb) from a region of historical mining
activities (Rainbow and Luoma, 2011) and strain Es524 is from a
Cu-contaminated coastal location in northern Chile (Caleta Palito),
receiving wastewater from a mine rich in Cu, Mn, and iron (Fe)
(Andrade et al., 2006). Oxidative stress and damage of the three
strains was assessed from measurements of H_2O_2, the levels of
thiobarbituric acid reactive substances (TBARS) and photosynthetic
pigments, and their antioxidant responses were determined from
the activities of the enzymes SOD, CAT and APX and the cellular
concentrations of ascorbate (ASC), dehydroascorbate (DHA), reduced
(GSH) and oxidized (GSSG) glutathione and phenolic compounds.

2. Materials and methods

2.1. Ectocarpus siliculosus strains, culture conditions and Cu treatments

E. siliculosus strain LIA08-4 (LIA; Culture Collection of Algae and
Protozoa (CCAP) accession number 1310/339) was isolated from
Lon Liath (56°56′N: 5°51′W), a location close to Arisaig, Scotland
with no history of anthropogenic impacts. Strain EcREP10-11 (REP;
CCAP 1310/338) was isolated from Restronguet Point (50°11′N:
5°3′W), near the mouth of Restronguet Creek in the Fal estuary,
a Cu-contaminated estuary in Cornwall, England; Cu_T concentra-
tions in sediments can be in excess of 2500 μg g^{-1} (Somerfield et al.,
1994). Strain Es524 (CCAP 1310/333) was isolated from Caleta Pal-
ito (26°15′S: 69°34′W) a Cu-impacted site in Chañaral, Chile; Cu_T
concentrations can be in excess of 1000 μg g^{-1} (Lee and Correa,
2005; Ramirez et al., 2005).

Strains were maintained in the laboratory as non-axenic unial-
gal cultures following the protocol outlined by Coelho et al. (2012).
Each strain was grown in 2 L polycarbonate bottles containing auto-
claved seawater enriched with Provasoli medium (Provasoli and
Carlucci, 1974) at 15 °C, 45 μmol m^{-2} s^{-1} photosynthetic active
radiation (PAR), 14:10 h light/dark cycle, and the cultures aerated
to prevent CO_2 depletion and maintain material in suspension.
Ten days (d) prior to experimentation strains were acclimatized to
the chemically defined synthetic seawater medium Aquil (Morel
et al., 1979) without the addition of the metal chelating agent
EDTA (Gledhill et al., 1999). Thereafter, 1 g fresh biomass (FW)
of each strain was transferred to individual polycarbonate flasks
containing 125 mL of Aquil medium and one of five concentra-
tions of CuSO$_4$·5H$_2$O, with three replicates per treatment. The algae

were exposed for 10 d to total dissolved Cu concentrations (Cu$_T$) of either 0 (no added Cu), 0.4, 0.8, 1.6, or 2.4 μM; these concentrations are equivalent to free Cu concentrations (Cu^{2+}) of 0.05, 21, 42, 85 and 128 nM, respectively (calculated using the Windermere Humic Aqueous Model [WHAM v7] and the stability constants in its default database; (Tipping, 1994; Varma et al., 2013). To prevent depletion of Cu^{2+}, as a result of the exudation of organic ligands from cells (Gledhill et al., 1999), the medium was replenished every 2 d. At the end of the experimental period biomass was briefly rinsed with sterile (autoclaved) seawater, immediately frozen in liquid nitrogen and stored at −80 °C to await analyses.

2.2. Quantification of hydrogen peroxide (H$_2$O$_2$)

Concentrations of H$_2$O$_2$ were determined according to Sergiev et al. (1997), with modifications for a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Frozen biomass (100 mg) was homogenized in 1 mL of 10% trichloroacetic acid (TCA) in a 1.5 mL centrifuge tube. Glass beads (5, 3 mm diameter) were added and the tubes vortexed for 5 min. The homogenate was centrifuged at 21,000 × g (Sanyo Hawk 15/05) for 10 min. Supernatant (50 μL) was added to each microplate well along with 150 μL of 50 mM potassium phosphate buffer (pH 7.0) and 100 μL of 1 M potassium iodide. The absorbance was determined at 390 nm. Concentrations between 0 and 3.2 mg mL^{-1} of H$_2$O$_2$ in 10% TCA were used as standards.

2.3. Quantification of thiobarbituric acid reactive substances (TBARS)

To quantify the end-products of lipid peroxidation, and thus provide an indication of damage to polyunsaturated fatty acids, the TBARS assay was performed according to Heath and Packer (1968), with some modifications. Algal biomass was extracted as described above and 200 μL of supernatant was mixed with 200 μL of 0.5% thiobarbituric acid (TBA) in 10% TCA, heated at 95 °C for 45 min in a water bath and then cooled to room temperature. The mixture (200 μL) was placed in a plate reader and the absorbance was measured at 532 nm. The standard 1,1,3,3, tetramethoxypropane, which breaks down to malondialdehyde (MDA) under the assay conditions, was used in a concentration range of 0 to 70 μM in 10% TCA.

2.4. Quantification of photosynthetic pigments

Chlorophylls a (Chla) and c (Chlc), and fucoxanthin (Fx) were extracted according to Seely et al. (1972) as follows. Fresh biomass (200 mg) was placed in a 1.5 mL glass test-tube to which 800 μL of dimethyl sulfoxide (DMSO) were added. After 5 min, samples were centrifuged for 30 s at 21,000 × g, the supernatants diluted with distilled water in a ratio of 4:1 DMSO:H$_2$O and absorbance measured spectrophotometrically (Jenway 7315). Pigment concentrations (nmol g^{-1} FW) were calculated using the following equations (where A_x = absorbance at λ_x):

$$[\text{Chl}a] = \frac{A_{665}}{72.5}$$

$$[\text{Chl}c] = \frac{(A_{631} + A_{582} - 0.297 A_{665})}{61.8}$$

$$[\text{Fx}] = \frac{(A_{480} - 0.722(A_{631} + A_{582} - 0.297 A_{665}) - 0.049 A_{665})}{130}$$

2.5. Preparation of extracts for enzyme assays

Extracts were prepared as described by Collen and Davison (1999). Frozen biomass (0.2 g) was homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.25% Triton X-100, 10% (w/v) PVP-40 and 1 mM EDTA. Glass beads (5, 3 mm diameter) were added and tubes vortexed for 10 min at 4 °C. Extracts were centrifuged at 21,000 × g for 5 min at 4 °C and the supernatant stored overnight at −20 °C or for 1 h at −80 °C to increase aggregation of biological membranes. Samples were centrifuged again at 21,000 × g for 5 min at 4 °C and extracts were stored at −80 °C.

2.6. Quantification of antioxidant enzyme activities

SOD activity was determined as described by McCord and Fridovich (1969), with modifications. The assay mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 18 mM cytochrome c and 0.1 mM xanthine. Xanthine oxidase was added to the assay mixture, just prior to its use, to give a final concentration of 0.0005 U mL^{-1}. Extract (50 μL) and 250 μL of total assay mixture were added to each well. The rate of change of absorbance at 550 nm was followed for 2 min. The SOD-insensitive rate was estimated using 10 μL of 1:9 diluted commercial SOD (Sigma Aldrich, G5389) in 100 mM phosphate buffer (pH 7) to obtain a rate that was between 5 and 10% of the rate in the absence of SOD. The SOD-insensitive rate was subtracted from all the data and the units were calculated by dividing the control rate by the rate in the sample, and subtracting 1 (Kuthan et al., 1986).

APX and CAT activities were determined as describe by Collen and Davison (1999). For APX, 50 μL of extract was added to 700 μL of 50 mM potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA and 0.5 mM ascorbate; H$_2$O$_2$ was added to a final concentration of 0.1 mM. The decrease in absorbance at 290 nm was monitored for 30 s. For CAT, 50 μL of extract was added to 700 μL of 50 mM potassium phosphate buffer, pH 7.0. The reaction was started by the addition of 11 mM H$_2$O$_2$ and the decrease in absorbance at 240 nm was monitored for 1 min; commercial CAT (Sigma-Aldrich, CAT100) was used as a positive control.

2.7. Quantification of ascorbate (ASC) and dehydroascorbate (DHA)

Concentrations of ASC and DHA were determined using ferric tripyridyl triazine (FRAP reagent), as described by Benzie and Strain (1999). Fresh biomass (300 mg) was placed in a mortar and ground to a powder in liquid nitrogen. A volume of 1.2 mL 0.1 M HCl was added, and the mixture centrifuged at 21,000 × g for 10 min at 4 °C. To measure ASC, 10 μL of extract was placed in each well of a 96-well plate, to which 290 μL of FRAP reagent was then added, and the absorbance measured immediately at 593 nm; with ASC standards (Fisher Scientific, 10061793), colour develops immediately and does not change significantly within the first minute. For total ascorbate, 500 μL of extracts were incubated for 1 h after adding 5 μL of 100 mM dithiothreitol. The reaction was stopped by addition of 5 μL (w/v) N-ethylmaleimide, and extracts were measured as described for ASC. Concentrations of DHA were calculated by subtracting values of ASC from total ascorbate.

2.8. Quantification of reduced (GSH) and oxidized (GSSG) glutathione

GSH and GSSG were measured as described by Queval and Noctor (2007). Extraction was performed as for ASC but neutralization was necessary by adding 5 M K$_2$CO$_3$ to obtain a supernatant with final pH ranging between 6.0 and 7.0. Total GSH was measured in a plate reader by adding 10 μL of neutralized supernatant to 290 μL of a mixture comprising 0.1 M sodium phosphate buffer pH 7.5, containing 6 mM EDTA, 0.34 mM NADPH, 0.4 mM DTNB, and 1 unit of GR (Sigma-Aldrich, G3664). Change is absorbance was
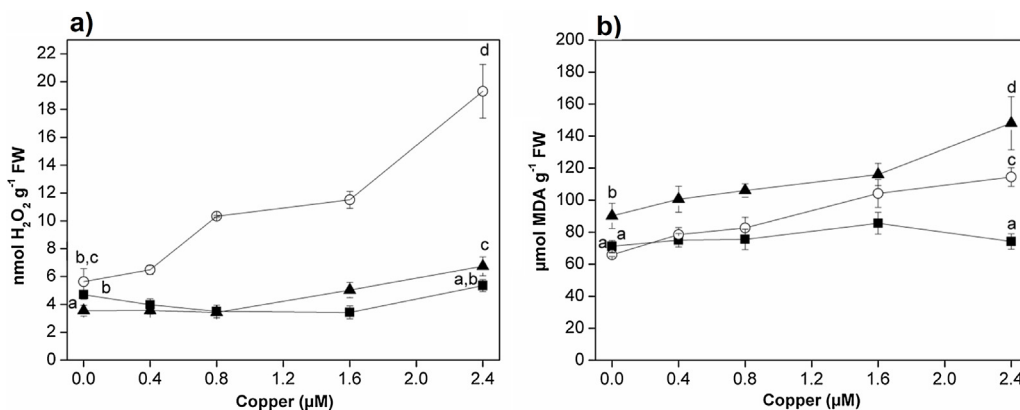
Fig. 1. Levels of H_2O_2 (a) and TBARS (b) in three strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu_T (128 nM Cu^{2+}) for 10 days. Strain LIA (open circle) is from a pristine site in Lon Liath, Scotland, REP (black triangle) is from a Cu-contaminated site in Restronguet Creek, England, and Es524 (black square) is from a Cu-contaminated site in Chañaral, Chile. Differences detected between 0 and 2.4 μM Cu_T ($P < 0.05$) are indicated by different letters. Error bars are ± SD, $n = 3$.

measured at 412 nm for 5 min. GSH (Sigma-Aldrich, G4251) was used as a standard at concentrations between 0 and 1 μM. To measure GSSG, 250 μL of neutralized supernatant was incubated for 20 min at room temperature after adding 5 μL of 4-vinylpyridine, and extracts then centrifuged at 21,000 × *g* for 5 min at 4 °C. GSSG was measured as for total GSH using GSSG (Sigma-Aldrich, G4501) as a standard at concentrations between 0 and 0.5 μM. GSH was calculated by subtracting 2GSSG from total GSH.

2.9. Quantification of total phenolic compounds

Total phenolic compounds were determined as described by Van-Alstyne (1995), with minor modifications. Fresh biomass (100 mg) was added to 15 mL tubes containing 5 mL of 80% methanol in distilled water. Glass beads (c. 10, 3 mm diameter) were added to aid the extraction. Tubes were placed in an Ika Laborthechnik (KS250) mixer and vortexed at 550 rpm for 24 h at 4 °C. Samples were centrifuged at 6000 × *g* at 4 °C for 10 min and the supernatant (12.5 μL) was added to 500 μL of 17% Folin–Ciocalteu reagent solution. After 5 min the solution was alkalinized with 250 μL of 1 M Na_2CO_3 solution and the samples were heated for 30 min at 50 °C in a water bath. The absorbance of the solutions was measured at 765 nm, using concentrations of phloroglucinol (Sigma-Aldrich, Y0000493) between 0 and 0.17% as standards.

2.10. Statistical analyses

The statistical package SPSS v.5 (IBM, USA) was used throughout. All data were checked for normality and homogeneity of variances with Shapiro–Wilk and Bartlett tests, respectively. Two-way ANOVA followed by post-hoc Tukey test (at 95% confidence) were performed to identify significant differences. To ease understanding of trends between strains in the figures, statistical indicators of significant differences were added only at 0 and 2.4 μM Cu_T.

3. Results

3.1. Cu-induced oxidative stress

In strain LIA (from a pristine location) the concentrations of H_2O_2 significantly increased with the concentration of Cu, from 5.6 nmol g^{-1} FW in controls to 19.3 nmol g^{-1} FW at 2.4 μM Cu_T. In REP (from a Cu-polluted location, England) there was a small, but significant, increase only at 2.4 μM Cu_T (3.5 to 5.5 nmol g^{-1} FW), and in Es524 (from a Cu-polluted location, Chile) no significant changes were observed (Fig. 1a). There was also no change in TBARS

levels with Cu exposure in Es524, whereas increases were apparent in the other two strains (Fig. 1b). In LIA, significant increases in TBARS levels were concentration dependent up to 2.4 μM Cu_T, whereas in REP a significantly higher level of TBARS, compared with controls, was measured only at 2.4 μM Cu_T (from 90.2 to 148 mmol g^{-1} FW).

3.2. Cu-induced changes in photosynthetic pigments

In LIA the concentrations of both chl*a* and chl*c* decreased significantly at the highest treatment level. There was more chlorophyll in REP than Es524, except at 2.4 μM Cu_T, but concentrations did not change significantly with Cu exposure, although a significant decrease in chl*a*, and increase in chl*c*, between controls and 2.4 μM Cu_T were measured in REP (Fig. 2b,c). In LIA, fucoxanthin content decreased significantly at concentrations above 0.8 μM Cu_T, from 300 to 200 mmol g^{-1} FW, whereas in Es524 concentrations increased between controls and 2.4 μM Cu_T, to 500 mmol g^{-1} FW. In REP, concentrations of about 500 mmol g^{-1} FW were measured with no significant changes between Cu treatments (Fig. 2d).

3.3. Cu-induced activation of antioxidant enzymes

The activities of the three enzymes under Cu exposure were strain and treatment dependent (Fig. 3). In REP under control conditions, the activity of SOD was significantly higher than that of the other two strains, and did not change significantly with increasing Cu. In LIA, significant increases from levels in controls (2.4 U g^{-1} FW) were observed but only at the two highest Cu concentrations (19.7 U g^{-1} FW at 2.4 μM Cu_T), whereas in Es524 there was a significant increase from 5.8 U g^{-1} FW in controls to 25.7 U g^{-1} FW at 0.4 μM Cu_T and did not change thereafter (Fig. 3a). Only in Es524 did the activity of CAT increase significantly with Cu exposure, from 0.06 to 0.16 μmol H_2O_2 g^{-1} FW min^{-1} in controls and 2.4 μM Cu_T, respectively (Fig. 3b). In all three strains increases in activity of APX were concentration dependent and at concentrations greater than 0.8 μM Cu_T there were no significant differences between strains (Fig. 3c).

3.4. Cu-induced changes in antioxidants

Changes in the concentrations of total ascorbate were strain and treatment dependent, and relate to specific modification in ASC and DHA content (Fig. 4a–c). Concentrations of ASC were significantly higher in Es524 than REP under all treatments, with levels increasing significantly between those of controls and 2.4 μM Cu_T (Fig. 4b).
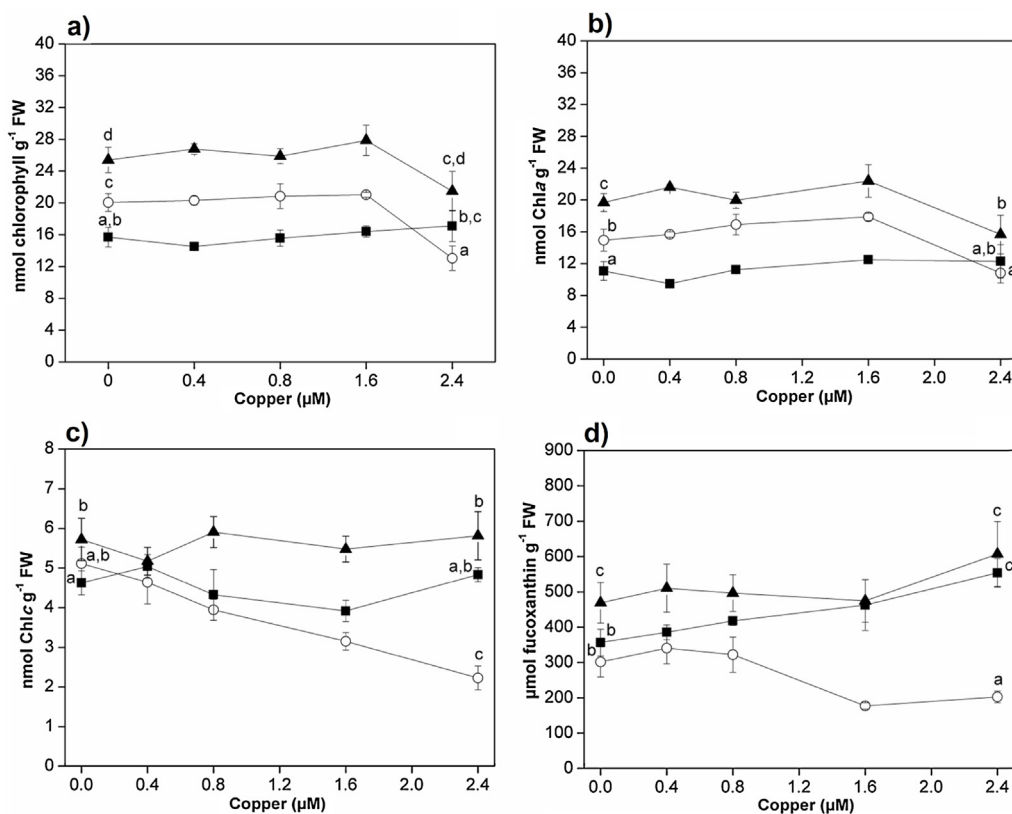
Fig. 2. Concentrations of total chlorophyll (a), chlorophyll *a* (b), chlorophyll *c* (c), and fucoxanthin (d) in three strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu$_T$ (128 nM Cu^{2+}) for 10 days. Strain LIA (open circle) is from a pristine site in Lon Liath, Scotland, REP (black triangle) is from a Cu-contaminated site in Restronguet Creek, England, and Es524 (black square) is from a Cu-contaminated site in Chañaral, Chile. Differences detected between 0 and 2.4 μM Cu$_T$ ($P < 0.05$) are indicated by different letters. Error bars are ± SD, $n = 3$.

In REP, there was a small, but significant, increase in DHA with Cu, whereas in Es524 levels remained the same up to 1.6 μM Cu$_T$ but then markedly increased at 2.4 μM Cu$_T$ (Fig. 4c). In LIA concentrations of ASC decreased significantly above 0.8 μM Cu$_T$, to a value of 80 nmol g^{-1} FW at 2.4 μM Cu$_T$ (Fig. 4b), and DHA concentrations significantly increased with Cu exposure (Fig. 4c).

Concentrations of total glutathione (GSH + GSSG) increased significantly in all three strains in a concentration dependent manner, with the largest increase (4.5 fold) occurring in Es524 (Fig. 4d), reflecting the change in GSSG (Fig. 4f). In both REP and Es524, GSH levels increased significantly with increasing Cu exposure whereas in LIA there was no significant change (Fig. 4e). As for GSSG, increases in LIA were Cu-dependent whereas in REP and Es524 increases were significant only at 2.4 μM Cu$_T$, with the greatest increase measured in Es524 (Fig. 4f). The changes in reduced and oxidized forms of glutathione are reflected in the glutathione redox ratios of GSH to GSSG (Table 1). In LIA the ratio decreased in a concentration dependent manner to values of less than 1, in REP the

ratio increased under low Cu and then declined thereafter to the value of controls, and in Es524 the ratio significantly increased at 1.6 μM Cu$_T$ and then declined to 1.2 at 2.4 μM Cu$_T$.

There was a general trend of increasing concentrations of phenolic compounds with Cu exposure, with significantly higher contents in Es524 and REP than LIA at all concentrations above 0.4 μM Cu$_T$ (Fig. 4g).

4. Discussion

The results presented in this study demonstrate that the three strains of *E. siliculosus* with different pollution histories display distinctive oxidative stress conditions and antioxidant responses. Specifically, the strain from the pristine site (LIA) had higher concentrations of H$_2$O$_2$, lower contents of photosynthetic pigments, ascorbate, glutathione, and phenolic compounds, and less activity of CAT and SOD at low Cu exposure levels than the strains isolated from Cu-impacted sites in Chile (Es524) and England (REP). Thus, strains chronically exposed to elevated concentrations of Cu exhibited more efficient antioxidant defence mechanisms but, interestingly, the precise nature of these mechanisms differed between these two tolerant strains. Higher activities of the enzymes SOD and CAT and greater production of ASC, GSH and phenolic compounds were found in Es524 compared with LIA. In REP, however, while there was a similar trend in the synthesis of antioxidant compounds, the activities of SOD and CAT did not increase with Cu exposure, although SOD activity was higher than in LIA in most treatments. These results for Es524 are in agreement with those for the brown seaweed *S. lomentaria*, also sampled from Caleta Palito, compared with individuals from the nearby pristine site of Caleta Zenteno (Contreras et al., 2005). Individuals from the

Table 1
Ratios of reduced and oxidized glutathione (GSH: GSSG) in three strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu$_T$ (128 nM Cu^{2+}) for 10 days. Strain LIA is from a pristine site in Lon Liath, Scotland, REP is from a Cu-contaminated site in Restronguet Creek, England, and Es524 is from a Cu-contaminated site in Chañaral, Chile. Errors are ± SD, $n = 3$.

Cu (μM)	Es524	LIA	REP
0	3.20 ± 0.69	5.9 ± 0.04	3.43 ± 0.50
0.4	3.15 ± 0.71	2.28 ± 0.08	5.37 ± 1.23
0.8	2.31 ± 0.51	1.13 ± 0.13	6.29 ± 0.73
1.6	4.99 ± 0.43	0.87 ± 0.12	3.97 ± 0.81
2.4	1.21 ± 0.30	0.70 ± 0.06	3.77 ± 0.10
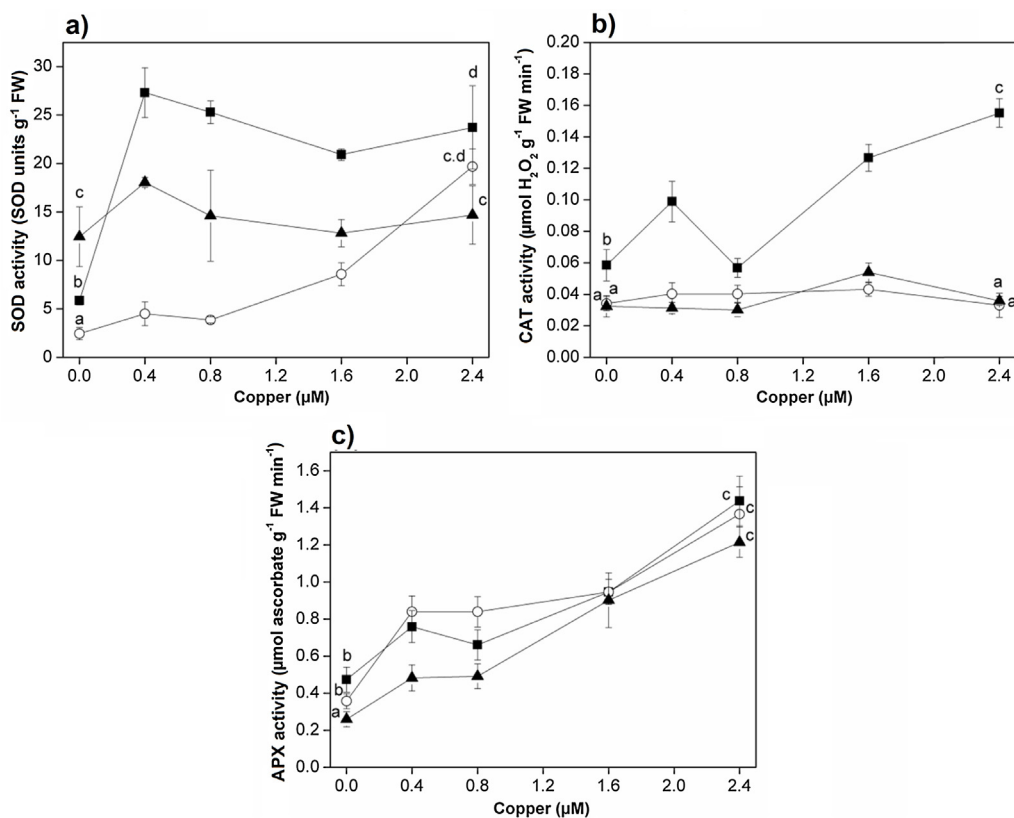
Fig. 3. Activities of the antioxidant enzymes SOD (a), CAT (b), and APX (c) in three strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu_T (128 nM Cu^{2+}) for 10 days. Strain LIA (open circle) is from a pristine site in Lon Liath, Scotland, REP (black triangle) is from a Cu-contaminated site in Restronguet Creek, England, and Es524 (black square) is from a Cu-contaminated site in Chañaral, Chile. Differences detected between 0 and 2.4 μM Cu_T ($P < 0.05$) are indicated by different letters. Error bars are ± SD, $n = 3$.

Cu-polluted site displayed an oxidative stress condition, reflected in an increase in TBARS levels, and countered by increased activities of CAT, and of APX and dehydroascorbate reductase (DHAR) (Contreras et al., 2005), antioxidant enzymes associated with the ascorbate–glutathione cycle (Noctor and Foyer, 1998). Additionally, their observations led them to suggest there was higher synthesis of ASC but not of either GSH or total phenolics (Contreras et al., 2005). Furthermore, *S. lomentaria* and *Lessonia nigrescens* cultivated *in vitro* with up to 1.6 μM Cu_T for 4 d showed increases in the activities of CAT, APX, glutathione peroxidase (GP) and DHAR as well as in levels of TBARS. However, lower levels of TBARS and greater enzyme activities were found in *S. lomentaria* than *L. nigrescens* indicating that the former species displayed more efficient antioxidant defences and less oxidative damage than *L. nigrescens* (Contreras et al., 2009). Therefore, Es524 and *S. lomentaria*, both isolated from the same Cu-contaminated location, share common Cu-induced antioxidant responses corresponding to increases in CAT and APX activities. However, the antioxidant defences in Es524 appear to be more effective than in *S. lomentaria* as there was no increase in TBARS under Cu exposure; this appears to be a result not just of increased activities of SOD, CAT and APX, but also of ASC, GSH and phenolic compounds. Contrary to the above, the green seaweed *U. compressa*, sampled directly from Caleta Palito, showed oxidative stress reflected in an increase in TBARS, despite an increase in APX activity, but not in CAT or other enzymes of the ascorbate–glutathione cycle such as DHAR and GR (Ratkevicius et al., 2003). In addition, *U. compressa* from Cu-impacted sites, compared to individuals from pristine locations, showed greater synthesis of ascorbate which was mainly accumulated as DHA, and a decrease in both GSH and phenolic compounds (Ratkevicius et al., 2003). In contrast to Es524 and REP, DHA levels in LIA were much higher than ASC at elevated Cu concentrations, suggesting

inhibition or damage to DHAR, which catalyses the reduction of DHA to ASC (Noctor and Foyer, 1998). ASC synthesis, that mainly occurs through the L-galactose pathway in plants and green macroalgae such as *U. compressa* (Mellado et al., 2012), did not appear to be affected in Es524 and REP since total ascorbate was always higher under Cu exposure than controls. In Es524 and REP, GSH and GSSG both increased with increasing Cu exposure, although concentrations of GSH were typically similar to or higher than GSSG in all treatments. On the contrary, in LIA concentrations of GSH did not change under Cu exposure while GSSG increased, resulting in a decline in the GSH/GSSG ratios to values below 1 at concentrations above 0.8 μM Cu_T. A similar change in the GSH/GSSG ratio has been reported for a resistant and sensitive strain of the green microalga *Scenedesmus acutus* f. *alternans* in response to nickel toxicity (Randhawa et al., 2001). Maintenance of GSH occurs by *de novo* synthesis through two consecutive ATP-dependent reactions involving γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthase (GS), and regeneration by recycling through reduction of GSSG in a glutathione reductase (GR) catalysed reaction (Mellado et al., 2012; Noctor et al., 2012). In *U. compressa* exposed up to 10 μM Cu_T for 7 d there was an increase in the activities of γ-GCS and GS, followed by high GSH content (Mellado et al., 2012), and increased activity of GR (González et al., 2010). Similarly, in roots of the vascular plant *Panax ginseng* exposed up to 10 μM Cu_T for 20 and 40 d, there was an increase in the activity of γ-GCS (Ali et al., 2006), and in *Lemna gibba* exposed to 8 μM Cu_T for 8 d, an increase in GR activity was measured (Babu et al., 2003). In contrast, the activity of GR decreased in *S. lomentaria* exposed up to 1.6 μM Cu_T for 96 h (Contreras et al., 2009). Comparing the two tolerant strains of *E. siliculosus*, both were efficient in maintaining GSH levels under Cu stress but, Es524 and REP appear to have different strategies to accomplish this.
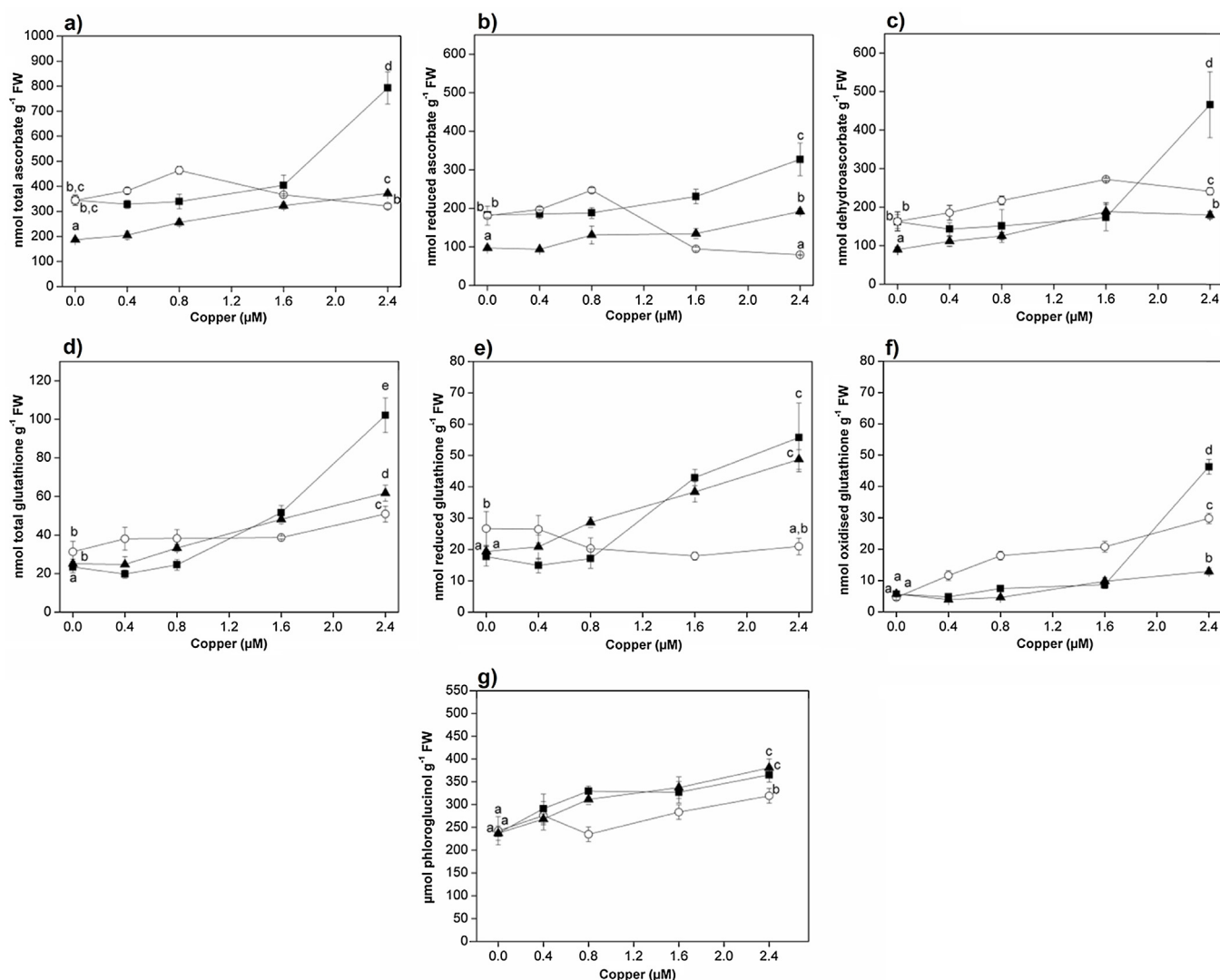
Fig. 4. Concentrations of antioxidants, total ascorbate (a), ASC (b), DHA (c), total glutathione (d), GSH (e), GSSG (f), and phenolic compounds (g) in three strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu_T (128 nM Cu^{2+}) for 10 days. Strain LIA (open circle) is from a pristine site in Lon Liath, Scotland, REP (black triangle) is from a Cu-contaminated site in Restronguet Creek, England, and Es524 (black square) is from a Cu-contaminated site in Chañaral, Chile. Differences detected between 0 and 2.4 μM Cu_T ($P < 0.05$) are indicated by different letters. Error bars are ± SD, $n = 3$.

Concentrations of GSH increased in a Cu dependent way in both Es524 and REP whereas GSSG was significantly higher in Es524 than REP at 2.4 μM Cu_T. These results suggest that *de novo* GSH synthesis is more efficient in Es524 than in REP while recycling of GSH, through the activity of GR, is better in REP than in Es524.

Interestingly, in REP there was less H_2O_2 but more TBARS than in LIA. This apparent inconsistency can be explained by the fact that SOD activity did not increase in REP, suggesting that $\bullet O_2^-$ anions may accumulate triggering oxidation of membrane fatty acids and an increase in TBARS levels. A similar phenomenon may also occur in *S. lomentaria* from a Cu-impacted site where the levels of H_2O_2 were lower than those of TBARS but, in this case, SOD activity was not measured (Contreras et al., 2005). Our data are also supported by the findings by González et al. (2010) who observed accumulation of $\bullet O_2^-$ and TBARS only after three days of exposure to Cu, and which continued until the end of the experiment on day 7. Accumulation of $\bullet O_2^-$ and TBARS was almost completely negated by diphenyleneiodonium (DPI), an inhibitor of flavin-containing enzymes such as those of mitochondrial complex I and II and chloroplast PSI, indicating that lipid peroxidation was a result of increases in organellar concentrations of $\bullet O_2^-$ in

U. compressa (González et al., 2010). Therefore, further studies are required on brown seaweeds to assess whether other ROS such as $\bullet OH$ radicals play secondary roles to $\bullet O_2^-$ anions in inducing lipid peroxidation under Cu stress as encountered in *U. compressa* (González et al., 2010, 2012a).

Another interesting observation is the significant decrease in the concentrations of photosynthetic pigments in LIA but not Es524 and REP under Cu stress, which may indicate that photosynthesis and growth would be impaired in LIA. In agreement with the latter, Nielsen and Nielsen (2010) observed that individuals of *F. serratus* under 2 μM Cu, for 12 d had significantly lower contents of both Chl*a* and Chl*c*, which was accompanied by a decrease in both the maximum quantum efficiency of PSII and relative growth rates. It has been observed that Cu excess can induce photo-inhibition of PSII due to a deficiency in Fe which is essential for chlorophyll synthesis (Patsikka et al., 2002). Moreover, Kuepper et al. (2002) found that photo-inhibition in *E. siliculosus* can be caused by the substitution of Cu^{2+} for Mg^{2+} in the chlorophyll molecule which suppresses photosynthesis. As the decline in chlorophylls was mainly observed in LIA (and not Es524 or REP), the information suggests that, instead of exposure to high Cu directly interfering with

chlorophyll synthesis, damage was occurring *via* Cu-mediated ROS excess. Furthermore, a proteomic analysis on strains Es32 (from a pristine site) and Es524 exposed to Cu stress, showed that the latter had higher levels of proteins corresponding to the Mn-stabilizing protein component of PSII and a fucoxanthin-Chl*a*/Chl*c*-binding protein, both of which protect PSII (Ritter et al., 2010). Thus, it is conceivable that Chl*a* and Chl*c*, as well as fucoxanthin, are protected against Cu-induced oxidative stress by these, or similar, over-expressed proteins in Es524 and REP but not, or at least to a lesser extent, in LIA. On the other hand, fucoxanthin, the main xanthophyll and light harvesting pigment in brown algae, has been shown to have strong antioxidant properties *in vitro*, and capable of quenching more $^1O_2$ and $\bullet OH$ than β-carotene (Mikami and Hosokawa, 2013; Sachindra et al., 2007). However, despite this observation, nothing has been described as to the importance of fucoxanthin as a ROS scavenger *in vivo*. Therefore, it is interesting to observe the increased fucoxanthin with Cu treatment in Es524 and high levels in REP under all Cu treatments. This might suggest that fucoxanthin is functioning as antioxidant in chloroplasts, further supported by the fact that in neither strain did the total chlorophyll concentrations alter significantly with exposure to Cu.

The evolution of metal-tolerant ecotypes is well documented in higher plants but there are very few examples of such in marine macroalgae, including brown seaweeds, although there is evidence for higher tolerance in populations inhabiting polluted compared with non-polluted sites, which might be an inherited characteristic. For example, it has been observed that populations of the brown seaweed *F. serratus* naturally exposed to elevated Cu^{2+} levels displayed higher growth rates in adults and their offspring, lower Cu accumulation and greater photosynthetic efficiency than individuals from pristine sites (Nielsen et al., 2003b). While it is evident that metabolic alterations are responsible for differential stress response and the development of tolerant ecotypes (Sordet et al., 2014), here we have found that two Cu-tolerant populations of *E. siliculosus*, from Chile and England, also display distinctive defence strategies, as manifested in their differential antioxidant responses to elevated Cu concentrations. The differences observed in the tolerant *E. siliculosus* strains Es524 and REP may be genetically inherited, a hypothesis supported by the high genetic diversity that characterizes *E. siliculosus* as a complex cryptic species (Dittami et al., 2011). However, such intra-specific differences in response could also be related to epigenetic changes due to chronic exposure to Cu in their natural environments. In this respect, it has been shown that plants of *Arabidopsis thaliana* exposed to high concentrations of Cu, Ni and Cd displayed a progressive incremental increase in tolerance over three to five generations and that genetic homologous recombination steadily increased after three generations (Rahavi et al., 2011).

The reasons for the divergent antioxidant responses observed between the two tolerant strains probably relate to the prevailing environmental conditions from where they were isolated. Although higher total Cu levels have been measured in Restronguet Creek (REP) than in Caleta Palito (Es524), with sediment concentrations up to 2500 (Somerfield et al., 1994) and $1000 \mu g\,g^{-1}$ Cu_T (Ramirez et al., 2005), respectively, the environments of the two locations are very different. While Caleta Palito is a fully-saline rocky-coastal site influenced mainly by tide and currents (Ramirez et al., 2005), Restronguet Creek is within an estuary, influenced by tidal flow and freshwater inputs with high organic content from the Carnon River (Rainbow et al., 2011; Titley et al., 1987). Dissolved organic matter alters metal speciation and can reduce bioavailability to algae (DePalma et al., 2011; Rainbow et al., 2011); therefore, the degree of tolerance needed for REP to survive may be less than that for Es524. Moreover, while very high Cu concentrations have been recorded in Restronguet Creek, the highest

measured levels are upstream of where REP was isolated (Grant et al., 1989). Support for this comes from a study on Cu-tolerance in the polychaete, *Hereis diversicolor* which was highest in samples obtained from the riverine-end and lowest from Restronguet Point at the mouth of Restronguet Creek (Burlinson and Lawrence, 2007). As *E. siliculosus* strain REP was isolated from Restronguet Point (http://www.ccap.ac.uk/strain_info.php?Strain_No=1310/338), it is highly likely that REP represents one of the least Cu-tolerant populations of *E. siliculosus* within Restronguet Creek. Further research is required to test this hypothesis.

Our investigation has demonstrated that while Cu excess induced high oxidative damage and overcame antioxidant defences in the *E. siliculosus* strain LIA from a pristine site, strains Es524 and REP, from Cu-polluted locations in Chile and England, respectively, showed robust, but divergent, antioxidant responses. Besides the mechanistic differences observed between non-tolerant and tolerant populations, this study highlights that even amongst the latter distinctive antioxidant strategies can develop, that most likely reflect the relative levels of stress imposed by the environmental conditions in which they inhabit.

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