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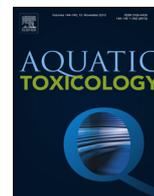
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Response differences between *Ectocarpus siliculosus* populations to copper stress involve cellular exclusion and induction of the phytochelatin biosynthetic pathway

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ABSTRACT

Some populations of brown seaweed species inhabit metal-polluted environments and can develop tolerance to metal stress, but the mechanisms by which this is accomplished are still to be elucidated. To address this, the responses of two strains of the model brown alga *Ectocarpus siliculosus* isolated from sites with different histories of metal contamination exposed to total copper (Cu_T) concentrations ranging between 0 and 2.4 μM for 10 days were investigated. The synthesis of the metal-chelator phytochelatin (PCs) and relative levels of transcripts encoding the enzymes γ -glutamylcysteine synthetase (γ -GCS), glutathione synthase (GS) and phytochelatin synthase (PCS) that participate in the PC biosynthetic pathway were measured, along with the effects on growth, and adsorption and uptake of Cu. Growth of strain LIA, from a pristine site in Scotland, was inhibited to a greater extent, and at lower concentrations, than that of Es524, isolated from a Cu-contaminated site in Chile. Concentrations of intra-cellular Cu were higher and the exchangeable fraction was lower in LIA than Es524, especially at the highest exposure levels. Total glutathione concentrations increased in both strains with Cu exposure, whereas total PCs levels were higher in Es524 than LIA; PC2 and PC3 were detected in Es524 but PC2 only was found in LIA. The greater production and levels of polymerisation of PCs in Es524 can be explained by the up-regulation of genes encoding for key enzymes involved in the synthesis of PCs. In Es524 there was an increase in the transcripts of γ -GCS, GS and PCS, particularly under high Cu exposure, whereas in LIA4 transcripts of γ -GCS1 increased only slightly, γ -GCS2 and GS decreased and PCS did not change. The consequences of higher intra-cellular concentrations of Cu, lower production of PCs, and lower expression of enzymes involved in GSH-PCs synthesis may be contributing to an induced oxidative stress condition in LIA, which explains, at least in part, the observed sensitivity of LIA to Cu. Therefore, responses to Cu exposure in *E. siliculosus* relate to the contamination histories of the locations from where the strains were isolated and differences in Cu exclusion and PCs production are in part responsible for the development of intra-specific resistance.

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1. Introduction

To counter the toxic effects of metals, photosynthetic organisms have evolved effective extra- and intra-cellular mechanisms for metal detoxification (Clemens, 2006). In algae, adsorption to cell

wall constituents and epiphytes (Correa et al., 1999; Holmes et al., 1991), exudation of organic substances (Gledhill et al., 1999), and altered permeability of the cell membrane (Brown and Newman, 2003) can constrain cellular uptake of metals. The superficial binding of metals can account for up to 95% of the total metal accumulated in freshwater species (Pawlik-Skowronska et al., 2004) and between <5% and 80% in marine macroalgae (seaweeds), depending on the metal, external concentration, species of alga, cell wall composition and the environmental conditions under which the algae are growing (García-Ríos et al., 2007; Vasconcelos and Leal, 2001).

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Metals enter cells mainly via energy-dependent transport across the plasma-membrane and therein, sequestration by peptides and polyphenols and synthesis of antioxidants and activation of antioxidant enzymes which alleviate metal-induced oxidative damage, have been implicated in the tolerance to metal stress (González et al., 2010; Mellado et al., 2012; Pinto et al., 2003; Sáez et al., 2015; Torres et al., 2008).

Glutathione, a low molecular weight thiol, is one of the most important antioxidants and metal chelators (Noctor et al., 2012). In its reduced form glutathione (GSH) is the primary pre-cursor of phytochelatin (PCs), small cysteine-rich oligopeptides (2–11 amino acids long) that chelate metals through their sulphhydryl groups (Cobbett, 2000; Torres et al., 2008). They are synthesised through two enzymatic pathways: (a) the synthesis of GSH by two consecutive ATP-dependent reactions involving γ -glutamylcysteine synthetase (GCS) and glutathione synthetase (GS), and (b) polymerisation of GSH to PCs by phytochelatin synthase (PCS) (Cobbett and Goldsbrough, 2002). Therefore, maintaining the equilibrium between synthesis and utilisation of GSH and production of PCs is critical to counteracting metal stress. Synthesis of PCs in response to elevated concentrations of metals such as cadmium (Cd), copper (Cu) and zinc (Zn) has been observed in different groups of photosynthetic organisms, but mostly in terrestrial plant taxa (Cobbett, 2000; Lee and Kang, 2005) and aquatic microalgae (Kobayashi et al., 2006; Scarano and Morelli, 2002). In contrast, metal-induced production of PCs in marine macroalgae (seaweeds) has been confirmed for relatively few species, with examples from all three phylogenetic groups (e.g. Gekeler et al., 1988; Kobayashi et al., 2006; Malea et al., 2006; Mellado et al., 2012). Most of these studies have emphasised the different abilities of species to synthesis PCs whereas only one study has reported on differences between populations within species (Pawlik-Skowronska et al., 2007), despite evidence for intra-specific variation in metal-tolerance in several species of seaweed (e.g. Brown et al., 2012; Brunetti et al., 2011; Nielsen et al., 2003b; Reed and Moffat, 1983).

The aim of this study was to investigate intra-specific responses to Cu-stress in the brown alga *Ectocarpus siliculosus*. Previous studies have provided evidence for a higher degree of Cu-tolerance in material isolated from Cu-enriched environments compared with that from uncontaminated sites, although the underlying tolerance mechanisms were not fully explained (Hall et al., 1979; Russell and Morris, 1970). *E. siliculosus*, and other members of the order Ectocarpales, are cosmopolitan filamentous seaweeds that exploit a wide-range of habitats, growing epiphytically, attached to hard substrata and as free-floating mats (Russell, 1967, 1983), and a range of environmental conditions from fully marine to freshwater (Charrier et al., 2008). Recently, *E. siliculosus* was proposed as a model organism for the study of brown algae, an ecologically and commercially important group of primary producers and bio-engineers in coastal waters and estuaries (Peters et al., 2004). Publication of the inventory of genes, following the sequencing of the entire genome (Cock et al., 2010), has provided new opportunities for post-genomic investigations, including furthering our understanding of the underlying mechanisms by which brown seaweeds respond to environmental stressors, including metal pollution (Ritter et al., 2010). The high levels of morphological, physiological and genetic variation within the species (Dittami et al., 2011), suggests the potential for differential cellular responses to Cu-stress. Therefore, to address this hypothesis the responses of two strains of *E. siliculosus* with different histories of Cu-exposure were assessed in laboratory cultures at environmentally realistic metal concentrations, from measurements of growth, metal accumulation, glutathione and PCs production, and transcript levels of genes encoding enzymes (GCS, GS, and PCS) involved in the synthesis of glutathione and PCs.

2. Materials and methods

2.1. *E. siliculosus* strains, culture conditions and Cu exposure

The two strains of *E. siliculosus* used in this study originated from locations with different histories of Cu contamination and have been maintained in culture since their isolation. Strain LIA08-4 (LIA; Culture Collection of Algae and Protozoa (CCAP) accession number 1310/339) was isolated in 2008 from north-west Scotland (Lon Liath, Arisaig; 56°56' N: 5°51' W), a location with no history of metal contamination. Strain Es524 (CCAP 1310/333) was isolated in 2004 from a Cu-contaminated area in northern Chile (Caleta Palito, Chañaral; 26°15' S: 69°34' W); Cu concentrations of up to 100 $\mu\text{g l}^{-1}$ in seawater and 1000 $\mu\text{g g}^{-1}$ in sediments have been measured at this location (Lee and Correa, 2005; Ramirez et al., 2005).

All glassware and polycarbonate bottles used throughout this study were acid-washed (0.1 M HCl) for 24 h and then rinsed in Milli-Q water (18 Ω). Chemicals were purchased from either Fisher Scientific (Loughborough, England) or Sigma-Aldrich (Gillingham, England).

Stock cultures of the algae were grown in 2 L polycarbonate bottles containing continuously aerated Provasoli Enriched Seawater (PES) (Provasoli and Carlucci, 1974), at 15 °C, 45 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, on a 14:10 light/dark cycle, following the protocols developed by Coelho et al. (2012). On production of sufficient biomass for experiments, the material was acclimatised for 10 d in the chemically defined seawater medium Aquil (Morel et al., 1979), but without the addition of a chelating agent (EDTA) as this can inhibit growth (Gledhill et al., 1999). Following acclimatisation, c. 1 g fresh biomass (FW) sub-samples were transferred to polycarbonate conical flasks containing 125 mL of Aquil and Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) to give total Cu (Cu_T) concentrations of 0 (no added Cu), 0.4, 0.8, 1.6 and 2.4 μM . The initial free (Cu^{2+}) concentrations were calculated by applying the Cu_T to the aqueous equilibrium speciation modelling programme, Windermere Humic Aqueous Model (WHAM v.6), as described by Nielsen et al. (2003b); concentrations of Cu^{2+} were 0.05 (control), 21, 42, 85, 128 nM, respectively. To avoid 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. The period of exposure was 10 d and there were 3 replicates per treatment for each strain.

2.2. Growth rates

The effect of Cu on growth was assessed volumetrically using the Wintrobe tube method described by Dring (1967). Known and equal volumes of biomass were inoculated in triplicate in polycarbonate conical flasks containing 50 mL of Aquil. Volumes (mm^3) were recorded prior to exposure (t_0) and after 10 days (t_1). Growth rates were calculated using the formula $[(V_{t_0}/V_{t_1})^{1/t} - 1]100\%$ ($\% \text{ d}^{-1}$) where V_{t_0} and V_{t_1} are volumes at the start and end of the exposure period (Yong et al., 2013).

2.3. Total and intra-cellular Cu concentrations

At the termination of the experiment and following removal of excess water, 40 mg FW subsamples of algae were either immediately frozen at -80°C or washed twice for 15 min in Aquil containing 10 mM EDTA to remove cell wall-bound Cu, thus distinguishing total from intra-cellular (non-exchangeable) fractions (Hassler et al., 2004), and then frozen at -80°C . Frozen biomass was freeze-dried for 24 h and digested in a microwave (MARSXpress; cycle of 34 min at 120–170 °C) using 2 mL of 70% (w/v) HNO_3 . Digested samples were diluted to 5 mL with milli-Q water and Cu concentrations were determined by ICP-MS (Thermo Scientific,

Table 1

Details of the selected genes: Gene ID from *Ectocarpus siliculosus* database (<http://bioinformatics.psb.ugent.be/webtools/bogas/search/in/Ectsi/current>) and accession numbers from GenBank database (<http://www.ncbi.nlm.nih.gov/pubmed/>).

Enzyme	Gene symbol	Gene ID	Accession number	Oligonucleotides – Forward–Reverse	PCR product size (bp)
γ -GCS	γ -GCS1	Esi0184.0033	CBJ30287.1	ACGACTCCATCAGCACCTTC CGACAAAGCCGAGTCTATCC	146
γ -GCS	γ -GCS2	Esi0250.0012	CBN79467.1	TGTCACACCCCTGTGAAGAA AGGGTAGTAGTCGCCCTCC	149
GS	GS	Esi0066.0082	CBN75075.1	GAGGCTGTTGGAGATGAACC CGGAAGTAAACCACCGCTAT	150
PCS	PCS	Esi0399.0022	CBJ32985.1	GACACACACCCCTCGAAGA GCACGAGGCTTCTCTCTTG	163

Hemel Hempstead, UK). External and internal calibrations of the instrument were achieved using Cu-certified standard solutions and Itrium (^{193}Ir) and Indium (^{115}In), respectively. Certified reference material (*Fucus* spp. IAEA-140) was treated in the same way as experimental material; the Cu concentrations were within 15% of certified values.

2.4. Total glutathione and phytochelatin (PCs) concentrations

Following exposure, biomass (200 mg FW) samples were frozen at -80°C and subsequently the non-protein thiols were extracted as described in de Knecht et al. (1994) and Pawlik-Skowronska et al. (2007). Samples were exhaustively extracted ice-cold with 1 mL of 5% 5-sulphosalicylic acid (SSA) containing 5 mM diethylenetriamino-pentaacetic acid (DTPA). Cells were disrupted by sonication (Vibracell ultrasonication processor) for 1 min (pulses of 10 s duration; output 300 W). The extract was centrifuged at 10,000 g for 20 min at 4°C , and the supernatant then syringe-filtered (0.20 μm pore size cellulose membrane, Minisart RC15, Sartorius).

Total non-protein thiols were determined with modifications as described in Gledhill et al. (2012). Thiols were reduced by adding 25 μL of 20 mM 2-carboxyethylphosphine hydrochloride (TCEP) to 250 μL supernatant, and the extract buffered to pH 8.2 with 160 μL of 200 mM HEPES. After 5 min incubation, 10 μL of a 100 mM monobromobimane (MBrB) was added followed by 465 μL of the HEPES/DTPA (pH 8.2). The derivatisation procedure was carried out in a dark room under dim red light conditions. After 15 min the reaction was stopped by the addition of 100 μL of 1 M methanesulphonic acid (99% purity, Sigma) and the extract stored in brown vials in the dark at 4°C .

Total glutathione and PCs were quantified by reverse-phase HPLC using a fluorescence detector at wavelengths of 380 nm excitation and 470 nm emissions. Separation was carried out using a 150 mm \times 21 mm C-18 HPLC column (Supelco) with a 3 μm particle size, and was achieved using a gradient programme of acetonitrile and 0.1% trifluoroacetic acid. The flow rate used was 0.2 mL min^{-1} . PC concentrations were derived from a calibration curve using reduced glutathione. Sample peaks in the chromatogram were identified from their relative retention times; GSH, PC2, PC3 and PC4 eluted at 11.7, 20.2, 23.7 and 26.7 min, respectively.

Since cadmium (Cd) is considered to be the most effective inducer of PCs (Pawlik-Skowronska et al., 2007), their preliminary detection and molecular structure was confirmed in E524 after exposure to 200 nM Cd^{2+} for 7 days by electrospray ionisation-mass spectrometry (ESI-MS). Exposure to Cd provided evidence for production of PCs, with an increase in the content of PC2, PC3, and PC4, relative to the controls (Supplementary Material 1).

2.5. Total RNA extraction and reverse transcription

Total RNA extraction was carried out with minor modifications, as described in Apt et al. (1995). All solutions were

prepared in water treated with diethylpyrocarbonate (DEPC), to inactivate RNase. Biomass (100 mg FW) was incubated in freshly prepared extraction buffer (0.1 M Tris/HCl pH 9.5, 1.5 M NaCl, 20 mM EDTA, 2%, w/v CTAB, 3%, w/v PVP-40), and 1% β -mercaptoethanol then added before extraction. Samples were incubated for 30 min at 10°C , one volume of chloroform/isoamyl alcohol (24:1, v/v) was added and then samples were shaken vigorously for 1 min. Following centrifugation at 21,000 \times g for 20 min at 4°C , the upper phase was transferred to a new tube, mixed with 0.2–0.3 V of absolute ethanol and 1 V of chloroform, and shaken vigorously for 5 min. The upper phase was recovered by centrifugation (13,000 rpm, 20 min) and the nucleic acid was precipitated overnight at -20°C using 0.1 V of 3 M sodium acetate (pH 5.2) and 0.6 V of ice-cold isopropanol. The pellet was recovered by centrifugation at 21,000 \times g for 20 min at 4°C , then washed with 75% ethanol, air dried and re-suspended in DEPC water. The RNA was treated with 30 U of DNase I recombinant, RNase free (Roche Diagnostic Mannheim, Germany) for 15 min at 37°C . This was followed by standard phenol-chloroform extraction and ethanol precipitation. The RNA pellet was dissolved in DEPC water. Quality and quantity of RNA was verified using a Nano-Drop[®] spectrophotometer ND-1000. The integrity was checked by agarose 0.8% gel electrophoresis. About 2–3 mg of total RNA were retro-transcribed using a First Strand cDNA Synthesis Kit (Fermentas, Milan, Italy) according to the manufacturer's instructions.

2.6. Primer design

Gene sequences for γ -glutamylcysteine synthetase (γ -GCS), glutathione synthase (GS) and phytochelatin synthase (PCS) were taken from the BOGAS gene annotation system (Bioinformatics Gent Online Genome Annotation Service, <http://bioinformatics.psb.ugent.be/orcae/overview/Ectsi>). One gene annotated to code for PCS (PCS; CBJ32985.1), one for GS (GS; CBN75075.1) and two gene sequences for γ -GCS (γ -GCS1, CBJ30287.1 and γ -GCS2, CBN79467.1) (Table 1). For mRNA level analysis, *E. siliculosus* specific oligonucleotide primers were designed in the coding sequence using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed 11 January 2006). The primers targeted highly conserved motifs to maximise the likelihood of detecting the expression level of the selected genes. Each primer pair used was designed to obtain a final PCR product of about 150–160 bp length and was tested according to different parameters: (i) robustness, successful amplification over a range of annealing temperatures, (ii) specificity, the generation of a single significant peak in the melting curve, and (iii) the consistency of highly reproducible C_T values within the reactions of a triplicate. The average efficiency of the primer pairs used ranged between 0.95 and 1.0. Alpha tubulin was selected as the housekeeping gene for chemical stress, as suggested by Le Bail et al. (2008).

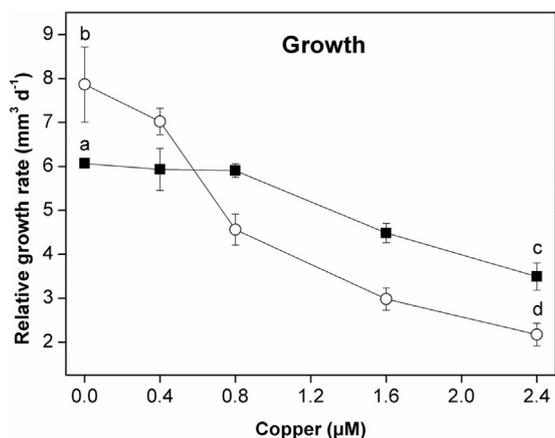


Fig. 1. Growth rates of two strains of *E. siliculosus* exposed up to 2.4 μM Cu_T . Strain LIA (circle) is from a pristine site, and Es524 (square) is from a Cu-contaminated site. Different letters represent significant differences at 95% confidence interval. Error bars are $\pm\text{SD}$ ($n=3$).

2.7. Quantification of gene transcripts by real time PCR

All genes were quantified on the same batch of cDNAs, to minimise experimental variation that could be due to cDNA synthesis. The quantitative RT-PCR reaction was performed with a StepOne Real-Time PCR Instrument (Applied Biosystems, Italy) in a 20 μL total volume containing: 10 μL 2 \times PowerSYBR Green PCR Master Mix (Applied Biosystems, Italy), 400 nM of each primer and 30 ng cDNA. Reactions were performed in triplicate with the following cycles: 95 $^\circ\text{C}$ for 10 min, 40 cycles of 95 $^\circ\text{C}$ for 15 s and 60 $^\circ\text{C}$ for 1 min. To test primer specificity, melting curve analysis (from 60 $^\circ\text{C}$ to 95 $^\circ\text{C}$ with an increased heating rate of 0.5 $^\circ\text{C s}^{-1}$) was performed after amplifications. The calculations for determining the relative level of gene expression were made using the cycle threshold (C_T) value, according to Livak and Schmittgen (2001).

2.8. Statistical analyses

Statistical tests were carried out using the software package SigmaPlot 12.0. The data were tested for homogeneity of variance and normality and then subjected to one- or two-factor analyses of variance (ANOVA). Differences between individual means were determined from Tukey's post hoc multiple range test at 95% confidence interval. The variation about means is plotted graphically as \pm the standard deviation (S.D.) of the mean.

3. Results

3.1. Cu-induced inhibition of growth and Cu accumulation

For both LIA (pristine site) and Es524 (polluted site) there was a general and significant decrease in growth rates with increasing Cu concentrations, but LIA was significantly more affected than Es524 at all concentrations above 0.8 μM Cu_T . Between controls and 2.4 μM Cu_T growth rates declined by 42% in Es524 and 72% in LIA. Under control conditions and at 0.4 μM Cu_T growth rates of LIA were significantly higher than those of Es524 (Fig. 1).

In both strains, the total Cu accumulated increased with Cu exposure, although in Es524 there was no further significant increase between 1.8 and 2.4 μM Cu_T . Significantly more Cu was accumulated by Es524 than LIA at 1.6 and 2.4 μM Cu_T , but the distribution of this Cu differed between strains (Fig. 2A–C). At lower concentrations (0.4 and 0.8 μM Cu_T) approximately 40–50% of the accumulated Cu was associated with the non-exchangeable (intra-cellular) fraction in both strains while at higher concentrations the

proportion of intra-cellular Cu decreased to approximately 30% in Es524 but did not change in LIA.

3.2. Cu-induced changes in total glutathione and PCs

In both strains the total glutathione pool significantly increased with Cu exposure. Except at 0.5 μM Cu_T , the concentrations of total glutathione in LIA4 were either higher or similar to those in Es524. Maximum mean concentrations were 479 and 378 nmoles g^{-1} (DW) for LIA and Es524, respectively (Fig. 3A). Total PC production was significantly higher in Es524 than LIA4A under all treatments, including the control. In Es524, concentrations of PC2 significantly increased from 10 nmoles g^{-1} (DW) in controls to a mean concentration of 55 nmoles g^{-1} (DW) above 0.8 μM Cu_T . In LIA exposure to Cu did not induce a significant increase in PC2, except at the highest exposure treatment with an increase from 20 to 30 nmoles g^{-1} (DW) between controls and 2.4 μM Cu_T (Fig. 3B). Longer chain-length PC3 was detected in Es524 but not LIA. The highest concentrations of PC3 were measured in controls (12.2 nmoles g^{-1} DW) and decreased significantly to a mean of c. 6.2 nmoles g^{-1} DW at concentrations higher than 0.4 μM Cu_T (Fig. 3C). No PC4 molecules were detected in any Cu treatment for either strain, but were present in Es524 when exposed to 0.2 μM Cd^{2+} for 7 days (Supplementary Material 1).

3.3. Cu-induced changes in the level of transcripts encoding γ -GCS, GS and PCS

At concentrations up to 0.8 μM Cu_T the relative level of transcripts encoding γ -GCS1 and γ -GCS2, the two isoforms of the enzyme required for the first step in GSH synthesis, were either the same in the two strains or significantly higher in LIA than Es524. In Es524 levels of both isoforms of γ -GCS increased with Cu exposure up to 2.4 μM Cu_T , whereas in LIA the levels of γ -GCS1 and γ -GCS2 transcripts increased until 0.8 μM Cu_T and then decreased at higher levels of Cu exposure (Fig. 4A and B). The highest levels of GS transcripts, the enzyme required for the second step in GSH synthesis, were measured in LIA under control conditions and at 0.4 μM Cu_T , and remained low at higher Cu_T . In Es524, the trend of increasing levels of GS transcripts with Cu exposure was only significant at 2.4 μM Cu_T (Fig. 4C). The level of transcripts encoding PCS, the enzyme responsible for PCs synthesis, did not change in LIA but increased markedly in Es524, especially at 2.4 μM Cu_T (Fig. 4D).

4. Discussion

The results presented here provide further evidence of intra-specific variation in the responses and tolerance to Cu exposure in the marine macroalga *E. siliculosus*. Several earlier studies documented different growth responses in strains that were related to the extent of Cu contamination in the environment from where they originated (Hall, 1980; Hall et al., 1979; Russell and Morris, 1970). More recently, Ritter et al. (2010) reported 70% cell death at 0.8 $\mu\text{mol L}^{-1}$ Cu_T in strain Es32 isolated from a non-contaminated site, compared with a similar level of inhibition only at 4 μM Cu_T in strain Es524 from a Cu-contaminated location. Our results are in general agreement with these findings; growth of Es524 was reduced by 42% at 2.4 μM Cu_T (equivalent to 128 nM Cu^{2+}) whereas in LIA, isolated from a pristine site, there was a 50% reduction at about 1.6 μM Cu_T (equivalent to 85 nM Cu^{2+}). The apparent greater sensitivity of Es524 in this study compared to Ritter et al. (2010) may be due to the bioavailability of free Cu^{2+} that would be influenced by the chemical composition of the growth media. Interestingly, under control conditions the growth rate of Es524 was significantly lower than that of LIA (8 versus 6 $\text{mm}^3 \text{d}^{-1}$), a result also found by Hall (1980) in one of their two culture conditions,

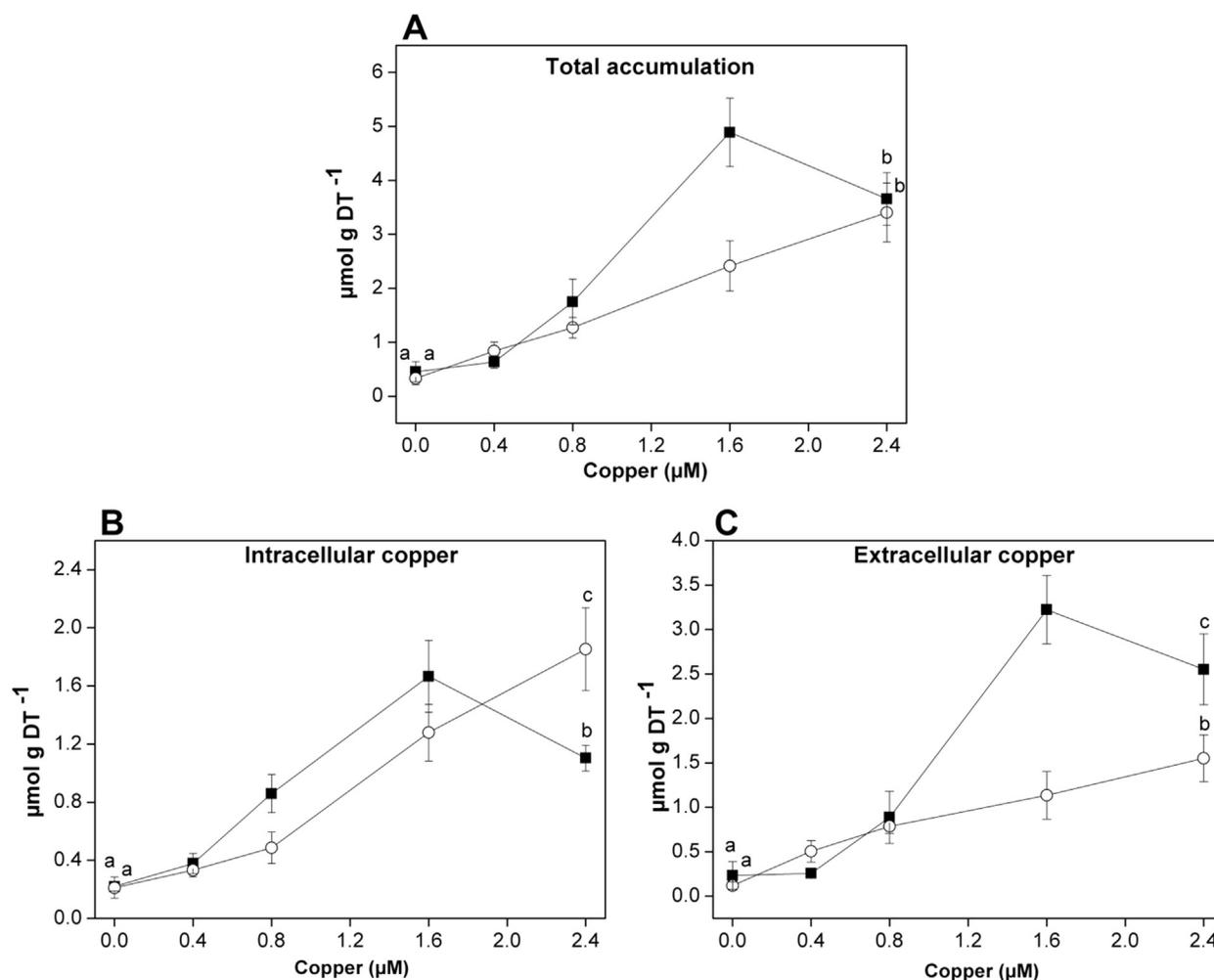


Fig. 2. Total copper accumulation (A), intracellular copper (B), and extracellular bound copper (C), in two strains of *E. siliculosus* exposed up to 2.4 µM Cu_T. Strain LIA (open circle) is from a pristine site, and Es524 (square) is from a Cu-contaminated site. Different letters represent significant differences at 95% confidence interval. Error bars are ±SD, n = 3.

but not commented on. A similar observation has been reported for the brown seaweed *Fucus serratus* (Nielsen et al., 2003a); in controls, rhizoid elongation rates of embryos derived from a Cu-tolerant population were significantly lower than those from a less polluted site. One explanation is that there is a 'cost' to metal tolerance. There is circumstantial evidence from studies on terrestrial plants that the 'cost' of tolerance is due to diverting energy resources away from supporting, for example, vegetative growth and reproduction in favour of maintaining tolerance. This has been termed the 'trade-off' hypothesis (e.g. Dechamps et al., 2008).

The accumulation of Cu was concentration dependent for both strains. However, of more significance is the finding that the distribution pattern of the accumulated Cu was dependent on exposure levels and differed between strains. At low Cu, approximately 50% of the accumulated Cu in both strains was associated with the cell walls and inter-cellular spaces (the exchangeable fraction), and at higher concentrations this portion increased to 70% in Es524. The effectiveness of metal adsorption in brown algae is related to the concentration and composition of alginic acid and sulphated polysaccharides. Carboxylic groups between guluronic (G) and mannuronic (M) acid segments of alginic polymers are the key targets for metal ions, and the ratio of M/G, which changes with environment condition, mediates the metal binding capacity of brown algae (Davis et al., 2003). Hall et al. (1979) proposed that Cu tolerance of *E. siliculosus* was due to exclusion of metals from

cells, although the evidence for such a conclusion is unclear. Here, unambiguous evidence is provided to support the view that exclusion of Cu from the cells of Es524 is, in part, responsible for a higher level of tolerance in this strain, evidenced by the higher growth rates at 1.6 and 2.4 µM Cu_T. These results highlight the importance of distinguishing between adsorbed and intra-cellular fractions of accumulated metals, as previously discussed by Vasconcelos and Leal (2001) and Andrade et al. (2006).

The ability of Es524 to maintain higher growth rates at cellular concentrations of Cu that inhibit the growth of LIA implies that there are effective intra-cellular defence mechanisms to reduce the toxic effects of the metal. The results obtained in this investigation provide the first evidence for the synthesis of phytochelatin (PCs) in a brown alga exposed to Cu and, moreover, for intra-specific variation in their production. Concentrations of PC2 increased with Cu exposure in Es524, but not in LIA. Furthermore, the longer chain dimers PC3 were detected only in Es524, although their concentration did not increase with Cu treatment. It is well established that PCs of longer chain-length more effectively chelate metals due to the availability of more binding sites (Cobbett, 2001). Mostly shorter length PC2 were produced, implying that these are sufficient to effectively bind Cu in Es524, at least within the range of exposure used in this study; whether longer chain PCs can be produced in Es524 at higher Cu concentrations requires further investigation. However, we provide evidence that longer chain PCs

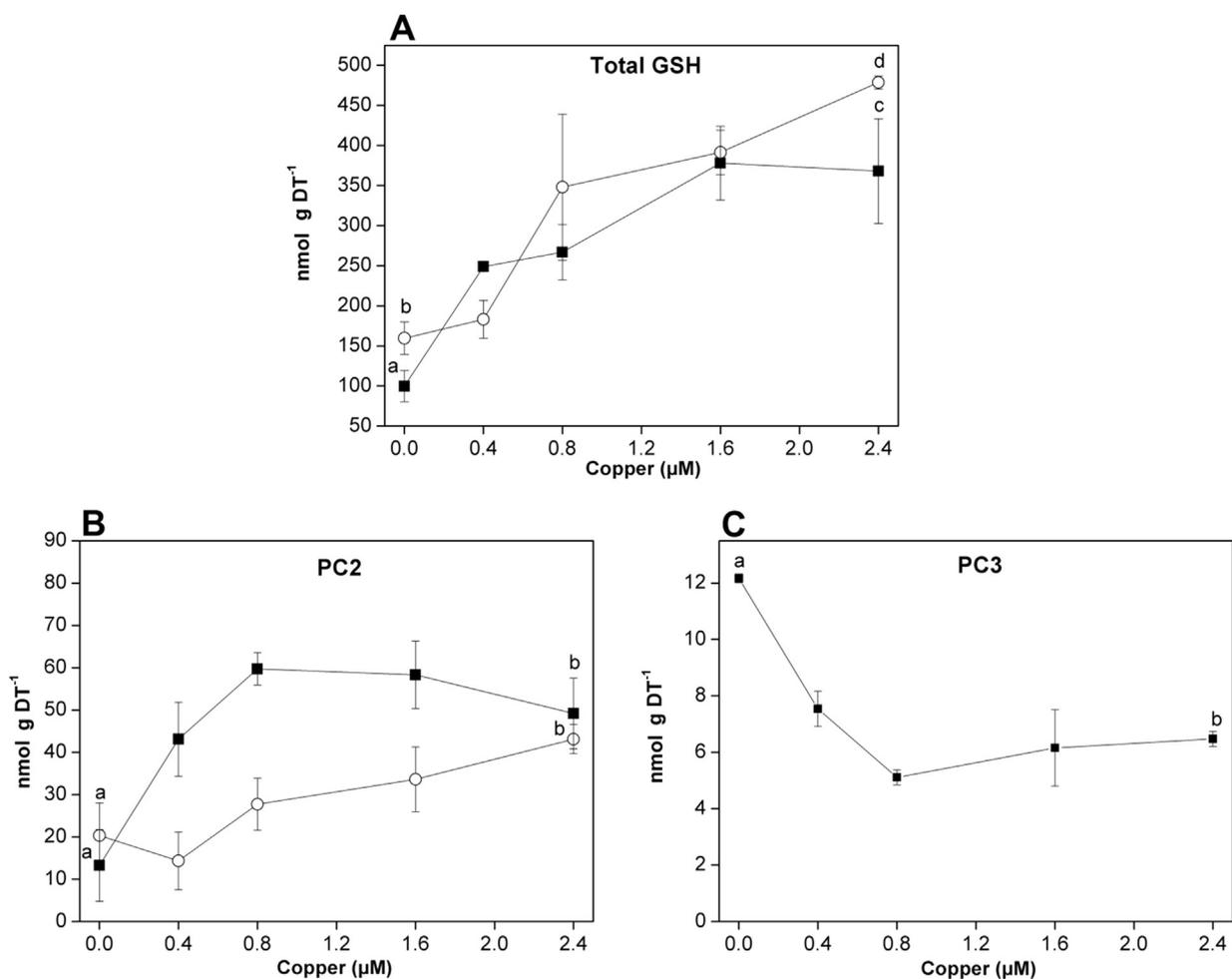


Fig. 3. Total glutathione (A), PC2 (B), and PC3 (C) concentrations in two strains of *Ectocarpus siliculosus* exposed up to 2.4 μM Cu_T. Strain LIA (open circle) is from a pristine site, and Es524 (square) is from a Cu-contaminated site. Different letters represent significant differences at 95% confidence interval. Error bars are \pm SD, $n = 3$.

can be produced in Es524, at least when exposed to Cd, considered the strongest inducer of PCs (Cobbett, 2000, 2001). Increased concentrations of dimers PC2 and PC3, and PC4 were detected. Investigations on PCs induction under metal stress in brown algae are rare, making comparisons difficult. However, PCs production (PC2–PC4) has been observed in the inter-tidal brown seaweed, *Fucus serratus* when exposed to 1 mgL⁻¹ of Cd for up to 11 d Pawlik-Skowronska et al. (2007). These authors have also provided evidence for greater production of GSH and PCs in *F. serratus* and *F. vesiculosus* sampled from a metal (including Cu) polluted site compared with material from uncontaminated locations in south-west England. Interestingly, the pattern of increases in GSH and PCs differed between the two *Fucus* species, and there were also marked differences amongst green and red seaweed species, despite growing under identical environmental conditions. The published information on seaweed taxa, including browns, suggests that production of GSH and PCs and, their overall significance within the metal stress machinery can be highly variable. Our results provide additional evidence that GSH-PCs production can also be population-specific, allowing some species to thrive within metal-enriched environments.

A similar pattern of expression was observed for the genes measured in the pathway of GSH-PCs biosynthesis, γ -GCS1, γ -GCS2, GS, and PCS; however, the trends were different between strains. For example, the expression of PCS increased strongly in Es524 with Cu exposure; for example, at 150 μgL^{-1} Cu there was a 1500-fold increase of the expression of PCS. In contrast, up-regulation

of PCS in LIA showed a cut-off threshold at 0.8 μM Cu and then suffered down-regulation at higher levels of Cu exposure. The pattern of increase in the expression of GCS and GS in Es524 under chronic Cu exposure provides evidence that this strain is likely to produce more GSH than LIA, one of the main intra-cellular antioxidant (Noctor et al., 2012) and the precursor of PCs (Cobbett, 2001; Mellado et al., 2012). The induction of PCS has previously been recognised as an important, and conserved, mechanism for the synthesis of PCs in a wide range of organisms, including algae, vascular plants, fungi and some invertebrates (see Rea, 2012). Related information is lacking for other brown algae but comparisons can be made with other photoautotrophs under metal stress. For instance, Zhao et al. (2010) transformed a γ -glutamylcysteine synthetase (GCS) cDNA into individuals of the non-tolerant plant *Agrostis palustris* with a γ -GCS gene, and when exposed to 15 mM Cd for 5 d there was stronger up-regulation of γ -GCS and PCS in transgenic individuals than wild-types. Associated with this up-regulation was an increased production of PCs while levels of lipid peroxidation were lower than in wild-type individuals. These results demonstrated that PCs play an important role in intra-cellular metal detoxification in the transgenic plant, making it more tolerant to Cd-mediated oxidative damage than the wild-type. Similar results have been gathered by Mellado et al. (2012), who observed stronger induction of γ -GCS and GS in the green alga *Ulva compressa* under 10 μM Cu_T for 7 d, as evidenced by increases in the activities of both enzymes. Our findings suggest that in terms of the expression of genes encoding for the enzymes γ -GCS, GS, and PCS, strain Es524 is in a better

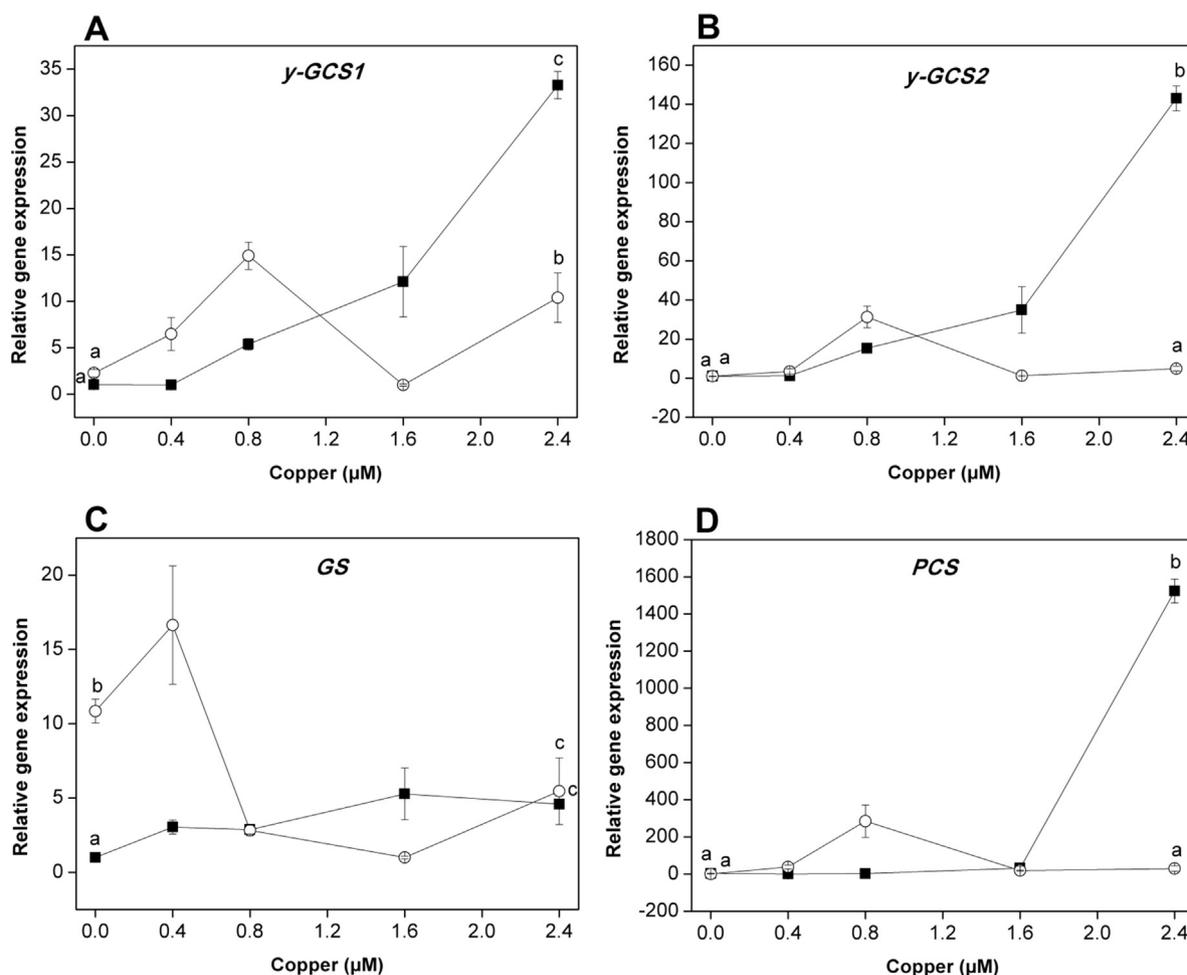


Fig. 4. Level of transcripts encoding for γ -GCS1 (A), γ -GCS2 (B), GS (C), and PCS (D), in two strains of *E. siliculosus* exposed up to 2.4 μ M copper. Strain LIA (open circle) is from a pristine site, and Es524 (square) is from a copper polluted site. Different letters represent significant differences at 95% confidence interval. Error bars are \pm SD, $n = 3$.

state of readiness to increase their activities, and can therefore synthesise more GSH-PCs than LIA under chronic exposure to Cu.

Our study provides evidence for a differential pattern of expression in γ -GCS1 and γ -GCS2 and GS genes between the two strains, and suggests that Es524 should produce more GSH. However, the concentrations of total glutathione (GSH + GSSG) increased with Cu exposure in both strains, following a similar trend. It has been observed that in situations of metal-mediated ROS excess, GSH is oxidised to GSSG and the ratio GSH/GSSG can be used as a biomarker of oxidative stress in photoautotrophs (Noctor et al., 2012). Our recent spectrophotometric analyses of GSH and GSSG concentrations in LIA and Es524 under similar Cu exposure (Sáez et al., 2015), indicate that while the quantity of total glutathione might be similar in the two strains under Cu stress, the percentage of GSH in the glutathione pool is significantly lower in LIA than Es524. The latter may also imply that there is less GSH available in LIA for PCs synthesis and polymerisation.

Another interesting observation is that PCs (including PC3 in Es524) were found in both strains under control treatments, with no added Cu. There is some evidence to suggest that PCs play other important roles than just as metal chelators, including participating in homeostatic regulation and storage of essential metals (Rea, 2012; Steffens et al., 1986; Tennstedt et al., 2009), and initiating the peptidic cleavage reactions of GSH conjugates leading to their cytosolic turnover in plants (Blum et al., 2007) and animals (Rigouin et al., 2013). Our results also agree with those reported by Pawlik-Skowronska et al. (2007), who identified PCs (PC2–PC4) in *F. serratus* in control treatments of Cd exposure experiments.

Compared with strain LIA, Es524 displays a combination of defence mechanisms to counter the toxic effects of Cu and permitted higher RGRs to be maintained at the highest concentrations of bioavailable Cu used in this investigation. These include, exclusion of Cu by efficient extra-cellular adsorption, stronger up-regulation of genes encoding enzymes intimately involved in the biosynthetic pathways of GSH-PCs, greater production of PCs with higher levels of polymerisation (PC2 and PC3) and an effective antioxidant response (Saez et al., in press), all of which means *E. siliculosus* can thrive in the highly Cu-impacted environment of Chanaral in Chile.

This is the first study to analyse concurrently, metal exclusion, intra-cellular metal content, glutathione and PCs concentrations, and the expression of genes encoding the enzymes catalysing GSH-PC synthesis in brown algae. Our results support the view that following a long history of exposure, strain Es524 has undergone genomic and/or epigenetic modifications, resulting in enhanced tolerance to Cu. These findings help to improve our understanding of the mechanisms involved in environmentally-induced inter-population responses to metal stress in an ecologically and economically important group of marine macroalgae.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2014.12.009>.

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