Physiological, biochemical, and molecular responses to copper stress in different strains of the model brown alga *Ectocarpus siliculosus*

By

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Abstract

Brown algae have been the focus of metal ecotoxicology research for over 60 years, mainly because of their high metal accumulation capacity and reputed resistance. Now that *Ectocarpus siliculosus* has been positioned as a model for the study of brown algae, and that the genome has been recently sequenced and annotated, new lines of research have been made possible on these ecologically and economically important organisms, including the field of ecotoxicology. Several strains of *E. siliculosus* have been collected and isolated from locations around the world, thus providing the opportunity to study inter-population differences in their responses to environmental stress. This investigation can be split into three main sections. In the first part Cu exposure experiments were carried out under laboratory conditions using three strains of E. *siliculosus*: Es524 from a Cu polluted location in Chile, REP10-11 from a metal polluted (including Cu) location in England and LIA4A from a pristine site in Scotland. Strains were exposed for 10 d to concentrations ranging between 0 and 2.4 µM Cu. We measured different parameters: relative growth rates; metal accumulation (extracellular and intracellular); phytochelatins and the expression of related enzymes; oxidative stress responses as manifested in lipid peroxidation and levels of H_2O_2, and levels of pigments; levels of antioxidants glutathione and ascorbate (in reduced and oxidised forms), and phenolic compounds; and the activity of the antioxidant enzymes superoxide dismutase, catalase, and ascorbate peroxidise. Strain Es524 was the most efficient in counteracting the effects of Cu stress as manifested by a combination of Cu exclusion production of metal chelators, upregulation of oxidative enzymes, and strong antioxidant metabolism. REP10-11 also showed effective Cu defences, especially related to glutathione-ascorbate interactions. LIA4A was the least tolerant strain, with metabolic defences significantly less effective against Cu exposure. In

part two a novel transplantation experiment was developed to compare responses in the field with those obtained in the laboratory. The study was carried out at a metal polluted and a low-impacted site in central Chile using strain Es524 (as in the laboratory experiments) and Es147, isolated from a low metal-polluted site in Chile. From the biomass, we conducted similar measurements of Reactive Oxygen Metabolism (ROM) as for the laboratory experiments described in the first part. In agreement with the laboratory experiments, strain Es524 displayed a higher resistance to metal stress. Because they behaved similarly between strains, the best suggested biomarker candidates for future assessments are metal accumulation, glutathione and ascorbate in reduced and oxidised forms, phenolic compounds, and the activity of superoxide dismutase. The method is simple, widely applicable in temperate environments, cost-effective, and provides a reliable representation of metal bioavailability in the environment. In the final part of the study a novel technique for the co-extraction of RNA and DNA, using a high pH Tris-HCl buffer, from small amounts of biomass of different strains of *E. siliculosus* was successfully developed. The extraction of nucleic acids from brown algae is considered to be difficult and the product is of poor quality due to the high concentrations of interfering secondary metabolites such as phenolics and polysaccharides. The protocol devised here provided high yields of pure RNA and DNA that are suitable for molecular analyses. This investigation provides new insights on metal stress metabolism in brown algae, and demonstrates that metal resistance is dependent on inherited defences developed over a long history of exposure. Furthermore, the good agreement between the results obtained in the laboratory with those from the field study confirms that the responses expressed under controlled laboratory conditions are representative of stress metabolism of *E. siliculosus* under natural conditions.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

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Publications:

- Greco, M., **Sáez, C.A.**, Bitonti, B., Roncarati, F., Brown, M.T. A simple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass *Ectocarpus siliculosus,* the model brown alga. PLoS ONE (*under review*).

- **Sáez, C.A.**, Gonzalez, A., Contreras, R., MoodyJ.,Moenne, A., Brown, M.T. A novel *in situ* method for assessing metal pollution using oxidative stress responses of the model brown alga *Ectocarpus siliculosus* (*In prep*).

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Signed ……..

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
LPX	Lipid peroxidation
Chl*a*	Chlorophyll *a*
Chl*b*	Chlorophyll *b*
Fx	Fucoxanthin
GSH	Reduced glutathione
GSSG	Glutathione disulphide
Asc	Ascorbate
DHA	Dehydroascorbate
MDHA	Monodehydroascorbate
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
GR	Glutathione reductase
GPX	Glutathione peroxidase
APX	Ascorbate peroxidase
CAT	Catalase
SOD	Superoxide dismutase

NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide

Chapter I

Literature review: metal stress metabolism in algae

1.1 Introduction

The oceans, which provide a vital sink for many trace metals and their compounds, are intimately involved in geochemical cycling. There is growing concern about the fact that natural cycling rates of many metals are being disrupted as a consequence of human activities (Ramade, 1987; Bruland and Lohan, 2006). For example, Patterson (1981; 1983) showed that lead concentrations in the atmosphere during the 1960s and 1970s produced a fiftyfold increase in ocean surface levels in the Central North Atlantic. Subsequent research revealed that during the 1980s lead concentrations decreased markedly, likely to be related to the replacement of leaded gasoline in the United States (Wu and Boyle, 1997). Several similar examples can be highlighted; for instance, metals zinc, copper and cadmium have increased in one to three orders of magnitude in aquatic natural fluxes due to anthropogenic inputs (Schindler, 1991). The most important anthropogenic routes for metal inputs into the sea are atmospheric and freshwater streams (GESAMP, 1990; Ridgway et al., 2003; OSPAR, 2010). Metal particles released into the air at ground level are mixed vertically and consequently contaminants may be transported many thousands of kilometres from where they were first released (see Marx et al., 2008; Vinogradova et al., 2008). For example, the high net accumulation of mercury, cadmium, vanadium and manganese in the Arctic ice cap and biota has been attributed to the atmospheric transport of these metals from temperate industrialised zones (Rahn and McCaffrey, 1979; Elmar R, 1981). In addition, domestic sewage and urban storm-water, as well as industrial and agricultural effluents represent an important input of metals to marine ecosystems (Perez et al., 2005; Li et al., 2009; Sarkar et al., 2011). The Quality Status Report 2010 (OSPAR, 2010) shows a better scenario in terms of pollution than past reports (see http://www.ospar.org/). The concentrations of metals in the North-East Pacific seem to

be decreasing as a result of improvements in technological advances and better regulations in industrial processes and waste management. Although atmospheric and waterborne inputs of metals declined significantly during the 1990s in OSPAR countries (Belgium, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, The Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and United Kingdom), after 1998 the reductions have been less notable as a consequence of technical and economic difficulties in the industrial sector. Despite improvements in environmental quality in the past two decades in the North-East Pacific, the concentrations of metals such as mercury, cadmium and lead have still been found to exceed the EU food standards in fish and shellfish in several locations and, moreover, present levels are likely to become a threat to marine life.

The accumulation of metals from solution by an organism is dependent on the bioavailability dictated by their dissolved state, mostly ionized. The bioavailability of metals, and therefore the equilibrium between ions and total dissolved metals, is governed by physico-chemical factors beyond the control of living organisms, such as salinity and the presence of metal-chelating substances (Nugegoda and Rainbow, 1989a; Nugegoda and Rainbow, 1989b). Some metals are essential for the metabolism; for example, Co, Cr, Cu, Mo, Ni, Fe, Mn, V and Zn are known to participate in metabolic reactions indispensable for maintaining homeostasis, including playing roles in several enzymatic complexes and proteins (Depledge and Rainbow, 1990; Falkowski and Raven, 2007; Yilmaz et al., 2010). It is known that marine organisms require essential metals within certain concentration limits, below these limits or in excess detrimental effects are expressed at different levels of biological organization (Rainbow, 1985). On the other hand, metals such as Pb and Al have no recognized role in any metabolic

process, and can be toxic even at trace levels (Farias et al., 2002). Many comprehensive reviews of metal concentrations and effects in marine organisms have been published (Bryan, 1984; Wagemann and Muir, 1984; Furness and Rainbow, 1990; Brown and Depledge, 1998; Das, 2000; Boran and Altnok, 2010; Eisler, 2010).

Chemistry of marine waters is always subject to variation, and hence chemical equilibrium is almost non-existent. To date, the vast majority of studies have used simple models to determine chemical speciation, with chemical equilibrium part of the assumptions (Hirose, 2006). More research in relation to the dynamics of aquatic environments is essential in order to better understand the relationships between metal speciation, bioavailability and uptake by living organisms (Galceran and van Leeuwen, 2004; van Leeuwen and Koster, 2004).

In the oceans, organic and inorganic compounds are dissolved in a concentrated salt solution, which can influence significantly the transport and bioavailability of metals (Hirose, 2006) (see Table 1.1). Until the 1970s the focus of metal speciation studies was mainly on the interactions between ionic metals and inorganic ligands, such as carbonate and hydroxide (Hirose, 2006), with limited information on relationships with organic ligands. It is now apparent that metals are found mostly as organic metal complexes (Gledhill and van den Berg, 1994; Ellwood and van den Berg, 2001; Vachet and Callaway, 2003). Such conformation of metal species have an important influence on geochemical cycles, and in their availability to marine organisms (Bruland et al., 1991; Hunter et al., 1997). Phytoplankton accounts for half of the primary production in the planet (NASA, 2009), and represents the most important source of organic matter in the ocean (Ghosal et al., 2002). Moreover, it has been recognized that the production of

organic metal-ligands by phytoplankton is the most relevant pathway controlling metal bioavailability and their interactions in marine environments (González-Dávila, 1995).

Table 1.1. Main metal complexes in the marine environment, from Scoullos and Pavlidou (2000) and Braspir and Butler (2009).

Inorganic		**Organic**	
		M_xL_y where L are organic ligands:	
		Carbohydrates	e.g. Glycol, cellulose
$M(aq)^{+n}$	where M: metal ion	Hydrocarbons	e.g. CH_4, C_6H_6
$MCl_x^{+(n-x)}$	x=1 to 4	Fatty Acids	e.g. $CH_3(CH_2)_{16}COOH$
$M(OH)_x^{+(n-x)}$	x=1 to 4	Humic Acids	
$M(SO_4)_x^{+(n-2x)}$	x=1 to 2	Siderophores	e.g. $C_{30}H_{27}N_3O_{15}$, $C_{42}H_{57}N12O_{16}$
$M(CO_3)^0$, $M(HCO_3)^+$		Fulvic Acids	
		Alcohols	e.g. CH_3OH, C_6H_6OH
		Porphyrins	e.g. Chlorophyll
		Sulfur compounds	e.g. CH_3SCH_3, RSH

The uptake of metals by marine organisms is most likely to occur in an ionic form dissolved in the water (Sunda and Guillard, 1976; Sunda et al., 1978; Sunda and Huntsman, 1983), and thus competition and equilibrium between metal ions and as inorganic/organic complexes are important factors controlling the process of uptake

(Sunda and Huntsman, 1998). Generally, concentrations of bioavailable metals in the water column are low because they precipitate as insoluble salts or to suspended particles, which eventually sink and are deposited in sediments. The vertical stratification of both ionic and complexed metals in the sediment varies, depending on redox processes. Beneath the upper oxic portion of sediments (2-5mm), where metals get deposited in (Kristensen, 2000), the environment becomes sub-oxic with mixtures of oxic complexes (e.g. Mn and Fe hydroxides) in equilibrium with a reduced dissolved phase (e.g. Fe(II) and MN(II)) (Simpson and Batley, 2003). Mediated changes in oceanographic conditions associated with factors such as water currents, upwelling processes, anthropogenic-mediated disturbances, and activity of benthic organisms, can influence 'recycling' of metals from the sediments to the overlying oxygenated seawater (Riedel et al., 1997).

1.2 Metal uptake and accumulation in brown algae

The bioavailability of essential metals is an important factor mediating algal development and growth, and as primary producers, it can have implications in trophic networks, ecosystems, and even globally (Brown and Depledge, 1998; Brown and Newman, 2003; Sáez et al., 2012b). For example, Fe forms part of hydrogenase complexes, which play an important role in the photosynthesis by reducing protons to hydrogen (Friedrich and Schwartz, 1993). Fe is also essential for the biosynthesis of chlorophyll, and its bioavailability is known to limit phytoplankton productivity in certain parts of the ocean (e.g. Lucas et al., 2007; Sugie et al., 2010). Several other examples can be highlighted; for instance, Cu and Mn are involved in enzyme activation and electron transport in photosynthesis, respectively (Falkowski and Raven,

2007). However, beyond certain threshold concentrations, essential and non-essential metals can represent a risk for the survival of brown algae. For example, Kupper *et al.* (2002) found that photo-inhibition in *Ectocarpus siliculosus* can be caused by the substitution of Mg^{2+} by Cu^{+2} in the chlorophyll molecule, incapacitating it for photosynthesis. Although the toxicity of essential and non-essential metals varies depending on concentration, bioavailability, the species of algae, and environmental factors, the general order of toxicity to algae is considered to be : Hg >Cu >Cd > Pb > Zn (Lobban and Harrison, 1994).

In brown algal cells, the uptake of Cu is generally accomplished through passive and active transport. Usually, passive transport is the first and most rapid process; metal ions diffuse into the cell in non-energy-dependent process influenced by aspects such as the permeability of the cell membrane and osmotic pressure (Gadd, 1988). Following the latter, uptake is continued by active transport in a much slower energy-dependent process; through intra-membrane proteins, metal ions are conveyed across the cell membrane towards the cytoplasm (Bates et al., 1982; Campbell et al., 2002). Although passive and active transport are important mechanisms regulating metal uptake, in a saturated environment, such as in a metal-polluted location, they are insufficient in order to avoid metal excess inside the cell; for example, reversible and irreversible intracellular binding and storage (Pinto et al., 2003). Because of their ecological importance and after millions of years of evolution, algae have developed different defence mechanisms in order to maintain metal homeostasis (Torres et al., 2008).

Several investigations have demonstrated that metal adsorption to cell walls can account for up to 95% of the total metal accumulation for some algae species (Mehta et

al., 2000; Freitas et al., 2008; Gonzalez Bermudez et al., 2011), and is considered to be the first barrier against metal excess in some metal-resistant algae (Lombardi et al., 2002). The algal cell wall is negatively charged due to the presence of several functional groups, including carboxyl (-COOH), hydroxyl (-OH), and sulphydryl (-SH) that are associated with different components of the cell wall, such as polysaccharides, peptidoglycan, proteins, and uronic acid. These functional groups readily bind metal cations, and their availability determines total metal binding capacity of cell walls (Wehrheim and Wettern, 1994; Romero-Gonzalez et al., 2001; Kaulbach et al., 2005; Hadjoudja et al., 2010). Cell wall chemistry and hence binding capacity varies between different algae classes (Kuyucak and Volesky, 1989). The main components of the cell walls of brown algae (Phaeophyceae) are cellulose (main structural skeleton), alginic acid (mannuronic and gluronic acids), and fucoidan (sulphated fucose) (Lee, 2008). Alginate can comprise between 10 to 40% dry weight (Percival and McDowell, 1967), and is considered to be the most important macromolecule involved in metal adsorption. The carboxylic groups between polymer segments and guluronic (G) and mannuronic (M) acids, are the main binding sites for metal ions (Haug, 1967; Puranik et al., 1999). The conformation of alginic acid, and more specifically changes in M/G ratios, is an important factor mediating metal binding capacity (Haug, 1967); M/G ratios vary between different species, between populations of the same species under different environmental conditions and between tissues in more complex species (Venegas et al., 1993; Davis et al., 2003). In a recent study, Millet et al. (2014) exposed *E.siliculosus* to 30 µM FeEDTA for 6 h, and then transferred to metal-free media for up to 168 h. They observed that Fe gets adsorbed to the cell surface during exposure; however, this Fe is eventually internalized by the cell after exposure. These authors proposed metal adsorption and internalization as an important mechanism for metal uptake under fluctuating environmental concentrations.

Competition between free metals ions (positively charged) for binding sites (negatively charged) can also occur, which can result in a decrease in the total binding capacity for specific metals (see Mehta and Gaur, 2005). Particulate material (non-living and living) on the surface of seaweeds can also bind metals. For example, epiphytic bacteria are able to bind and detoxify metals by means of adsorption to extracellular polysaccharides, metabolising and transforming metal ions into less harmful chemical forms (Riquelme et al., 1997; Goecke et al., 2010).

Brown seaweeds exude organic compounds that are able to complex metal ions, thereby reducing their bioavailability. Of these, phenolic compounds are considered one of the most important (Gledhill et al., 1999; Connan and Stengel, 2011). Phenolics are metal chelators and reducing agents, properties attributable to the electron donor capacity of the acidic hydroxyl groups (Ragan et al., 1980; Sueur et al., 1982). Large quantities of phenolics (specially phlorotannins) can be released and represents one of the most important sources of organic matter to the marine environment (Sieburth, 1969). Concentrations of 2.5 mg L^{-1} have been measured in coastal waters, even under normal environmental conditions (Ragan and Jensen, 1979; Swanson and Druehl, 2002; Shibata et al., 2006). The latter not only attributed to their metal chelation capacity, but also to other roles, such as repellent for bacteria and other epiphytic algae (Sieburth and Conover, 1965; Sakami, 1996), anti-grazing agent (Ank et al., 2013), and UV protection activities (Swanson and Druehl, 2002).

1.3 Production of intracellular metal chelators in brown algae

Phenolic compounds also represent an effective metal detoxification mechanism inside the cell in brown algae (Ragan et al., 1980; Pedersen, 1984). Intracellularly, phenolics are usually stored in spherical, membrane bound, bodies called physodes (Schoenwaelder, 2008). Gledhill et al. (1999) found that Cu concentrations in the extracellular media increased after exposing *Fucus vesciculosus* to up to 1000 nM Cu. The latter authors suggested that in addition to the exudation of phenolic compounds covered in the past section of this review, brown algae can also use phenolics to sequester metals intracellularly, and then transport them outside the cell.

Another important group of intracellular chelators are metal-binding polypeptides (Cobbett and Goldsbrough, 2002). These proteins can be separated into two main groups: short-chain polypeptides enzymatically synthesized called phytochelatins (PCs), and gene-encoded cysteine-rich metallothioneins (MTs) (Morris et al., 1999; Malea et al., 2006; Pawlik-Skowronska et al., 2007). PCs have the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, with a chain length "*n*" ranging between 2 and 11 units (Rauser, 1990; Cobbett and Goldsbrough, 2002). The metal binding capacity of PCs is given by their thiol groups, and have been observed mostly in photosynthetic organisms such as vascular plants (e.g. Rauser, 1995; Zenk, 1996), and algae (e.g. Perales-Vela et al., 2006; Pawlik-Skowronska et al., 2007; Tukaj et al., 2007; Osaki et al., 2008); however, they have also been described in fungi (e.g. Jaeckel et al., 2005) and in animals such as earthworms (e.g Liebeke et al., 2013). PCs are synthesised post-translationally from reduced glutathione (GSH) by the enzyme phytochelatin synthase (PCS) (Grill et al., 1989). The activation of this enzyme takes place when metal ions

bind to GSH complexes (Cobbett, 2000; Vatamaniuk et al., 2000; Osaki et al., 2009). There are several examples of PC induction in seaweeds upon exposure to metal stress in the laboratory (e.g. Gekeler et al., 1988; Skowronski et al., 1997; Hirata et al., 2001; Scarano and Morelli, 2002; Tsuji et al., 2002; Kobayashi et al., 2006; Scheidegger et al., 2011b), but only one published paper demonstrating induction in the field (Knauer et al., 1998; Pawlik-Skowronska et al., 2007). While it is known that the synthesis of PCs occurs specifically in the presence of metal ions, the importance of PCs within the overall metal defence machinery in algae is still not well understood. For example, Scheidegger et al. (2011a) exposed the unicellular green alga *Chlamydomonas reinhardtii* to short- and long-term exposure to Pb(II) and observed that during short-term exposure to increasing Pb(II) concentrations, levels of PCs also increased , during which period growth and photosynthesis were not inhibited. However, although PCs production increased during the longer-term of up to 72 h with a high level of polymerization, which increased until PCs with length chain of 5 GSH oligomers (PC5); growth and photosynthesis were inhibited by up to 100%. Pawlik-Skowronska et al. (2007) measured PCs in *Fucus* spp. in different locations of south-west England; the authors found that the highest levels of GSH (1550–3960 nmol SH g^{-1}dry weight) and PCs (200–240 nmol SH g^{-1} dry weight), with the greatest levels of polymerization (PC2-PC4), correlated with metal pollution history of the locations assessed and the concentrations of the metals As, Cu, Cd, Pb and Zn found in the thalli sampled (5.6-7.1 µmol g^{-1} dry weight). Published information suggests that PCs production is one of the first mechanisms activated when intra-cellular metal concentrations increase to toxic levels and that they are efficient during short-term exposure (Cobbett, 2000; Scheidegger et al., 2011a). However, during long-term exposure PCs synthesis can become insufficient to maintain metal homeostasis and other biochemical processes are necessary to counteract metal stress.

MTs are a family of cysteine-rich, low molecular mass proteins (5 to 10 kDa), synthesized in the Golgi apparatus. MTs can bind to metals ions through thiol groups of their cysteine residues with conserved motifs of cys-cys, cys-x-cys, and cys-x-x-cys, accounting for nearly 30% of the total amino acid residues (Robbins and Stout, 1992; Sigel et al., 2009). These oligopeptides are expressed in prokaryotes (e.g. Yoshida et al., 2002; Ruiz et al., 2011), animals (e.g. Trinchella et al., 2008; Kruczek et al., 2011), fungi (e.g. Munger and Lerch, 1985; Cicatelli et al., 2010), and photosynthetic organisms including plants (e.g. Hasegawa et al., 1997; Ma et al., 2003) and algae (e.g. Merrifield et al., 2006; Singh et al., 2008). The available information on algae is mainly restricted to the brown seaweed *Fucus vesiculosus*. The gene encoding MT was first identified in *F. vesiculosus* by Morris et al. (1999), describing a 1229 bp gene with a putative open reading frame of 204 bp, which encoded a protein of 67 amino acids with 24% cysteine residues, highly similar to the MT protein found in plants and some invertebrates. The authors also observed the induction of the MT gene by exposing the alga to Cu; later on, it was found that *F. vesiculosus* MT was also induced by other metals, such as As (Merrifield et al., 2004; Singh et al., 2008) and Cd (Merrifield et al., 2006). Although the properties of MTs are well known in other living organisms, their properties, the mechanisms and their specific contribution in the metal detoxification machinery of algae is still scarce.

1.4 Metals and reactive oxygen metabolism in algae: antioxidants and antioxidant enzymes

Reactive oxygen species (ROS) are highly reactive molecules due to their unpaired valence shell electrons (Smirnoff, 2005a). ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($\bullet O_2^-$), organic hydroperoxide (ROOH), alkoxy radicals (RO•), peroxy radicals (ROO•), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$), are signalling molecules and take part in important processes such as modulating gene expression and controlling the activity of protein complexes for defence purposes (Apel and Hirt, 2004). However, changes in environmental conditions can cause oxidative stress by intense ROS release, producing DNA damage, membrane injury and, in extreme conditions, apoptosis (Zuppini et al., 2010; Kumar et al., 2011).

As in all living organisms, metals take an active part in the reactive oxygen metabolism (ROM) of algae. They act as cofactors for several enzymes; for example, Mn, Zn, Ni, Cu and Fe are cofactors of the enzyme superoxide dismutase (SOD) (Wolfe-Simon et al., 2005; Wu and Lee, 2008). However, metal excess can also cause an over-production of ROS; for instance, by increasing the production of $OH^\bullet$ through Fenton reaction or by disrupting electron transport chains in photosynthesis in chloroplasts and respiration in mitochondria (Pinto et al., 2003; see Dring, 2005; Halliwell, 2006; Torres et al., 2008). In order to scavenge ROS and prevent oxidative damage, algae have developed metabolic defences, mainly based on the production of different antioxidants such as glutathione, ascorbate, phenolic compounds, and antioxidant enzymes like catalase, ascorbate peroxidise, superoxide dismutase and glutathione reductase.

Continuous thiol-sulfide interactions give glutathione its strong antioxidant characteristic and properties as a metal chelator (Noctor et al., 2012). In the presence of ROS, reduced glutathione (GSH) is easily converted into oxidized glutathione (GSSG) (Foyer and Halliwell, 1976; Grace and Noctor, 2000; Noctor et al., 2000), which is quickly reduced back to GSH by the enzyme glutathione reductase (GR) in a NADPH catalysed reaction (Halliwell and Foyer, 1978; Kataya and Reumann, 2010). The glutathione redox ratio, GSH:(GSH + 0.5GSSG), has been used as a biomarker of oxidative stress in several algal species after exposure to metals (e.g. Rijstenbil et al., 1994; Rijstenbil and Wijnholds, 1996; Ratkevicius et al., 2003; Malea et al., 2006; Kalinowska and Pawlik-Skowronska, 2010). To the extent of our knowledge there is no published information regarding glutathione ratios on brown algae under metal stress; however, for example, the terrestrial green alga *Geminella terricola* exposed to 0.5 µM of Cu decreased its GSH concentrations in 63% , causing significant decline in the GSH:GSSG ratio, compared with non-exposed alga (Kalinowska and Pawlik-Skowronska, 2010).

Ascorbate is an important scavenger of ROS. Reduced ascorbate (ASC) transfers a single electron to ROS to produce monodehydroascorbate (MDHA) in an ascorbate peroxidase (APx)-catalysed reaction. MDHA later dismutates to dehydroascorbate (DHA), the most stable form of oxidized ascorbate (Biaglow et al., 1997). The described pathways leading to ASC production include the synthesis from hexose sugars and from the reaction between DHA with GSH, which is mediated by the enzyme dehydroascorbate reductase (DHAR), basis of the glutathione-ascorbate cycle (See Potters et al., 2002; Smirnoff, 2005a; Smirnoff, 2005b; Noctor et al., 2012). In

different algae, experiments under controlled laboratory conditions and conducted in the field (polluted versus pristine environments) have shown a concomitant increase in the levels of ascorbate with increasing metal exposure (e.g. Ratkevicius et al., 2003; Bajguz, 2010; Maharana et al., 2010). For example , Ratkevicius et al. (2003) measured ASC and DHA levels in sampled in mining polluted areas and in other locations considered pristine and found that DHA increased significantly in relation to ASC in the green macroalga *Ulva compressa* from metal-polluted locations in Chile. The available evidence indicates that oxidation of ASC is an active mechanism against metal-induced over-production of ROS in algae.

Isoprenoids are organic molecules of general structure $(C_5H_8)_n$ that have important biological functions in photosynthetic organisms, such as light scavengers, providing photoprotection (e.g. carotenoids), and as antioxidants (e.g. tocopherols, tocotrienols and carotenoids). Carotenoids are a family of isoprenoids abundant in algae; a complete survey of the carotenoids present in different algae divisions has been recently published by Takaichi (2011). Carotenoids quench, principally, $^1O_2$ through a physical mechanism; they get excited by the transferred excess of energy of the radical into a triple state, and lose the extra energy in the form of heat (Murthy et al., 2005). It has also been proposed that carotenoids play a role in the chloroplastic reduction of oxygen, or chlororespiration, in the dark (Bennoun, 1998; Bennoun, 2001). In Chlorophyta, the genus *Dunaliella* has been the focus of carotenoid antioxidant research. For example, an increase in the levels of β-carotene under Cu excess in *D. tertiolecta* and *D. salina* (Nikookar et al., 2005), and the rise in total carotenoid levels in presence of high Hg levels in *D. tertiolecta* have been observed (Zamani et al., 2009). Similar patterns have been recorded in red algae; for instance, in *Gracilaria*

tenuistipitata, production of the major carotenoids violaxanthin, antheraxanthin, lutein, zeaxanthin, and β-carotene where observed to increase under Cu and Cd excess (Collen et al., 2003; Pinto et al., 2011). In brown algae, the antioxidant properties of carotenoids have been the focus of attention, especially in relation to food additives and in pharmacology. The major carotenoid in brown algae is the xanthophyll fucoxanthin, which is responsible for their brown colour (Bennett et al., 1969; Haugan and Liaaenjensen, 1994). Recently, fucoxanthin has received attention in the treatment of ailments such as cancer and obesity in humans due to its strong reducing and antioxidant characteristics (Masashi and Kazuo, 2007). It has been observed that fucoxanthin can quench $^1O_2$ and •OH *in vitro*, and has higher antioxidant capacity than α-tocopherol (Mikami and Hosokawa, 2013). Moreover, it has been recognized that within the fucoxanthin-chlorophyll a/c-binding protein complexes, fucoxanthin provides photo-protection against triple states of chlorophyll (Di Valentin et al., 2012). However, to date, there are no reports on the importance of fucoxanthin as a ROS scavenger for brown algal antioxidant metabolism.

Tocopherols and tocotrienols (vitamin E activity) are also isoprenoids recognized to have important antioxidant activity in the ROM of living organisms, and have been described in animals (e.g. Weber et al., 1997; Vieira et al., 2011), plants (e.g. Packer et al., 2001; Matringe et al., 2008), and algae (e.g. Mallick and Mohn, 2000; Durmaz et al., 2007; Sirikhachornkit et al., 2009), among others. Heterotrophic organisms acquire tocopherols and tocotrienols from photosynthetic organisms, as they are not capable of synthetizing these compounds (Gómez-Coronado et al., 2004). Different forms of tocopherols and tocotrienols are produced in algae, including the majority of cyanobacteria (Maeda and Dellapenna, 2007). They are characterized by a

hydrophobic isoprenoid tail and hydrophobic chromanol head, which can donate single electrons to ROS. They also react with $^1O_2$ by absorbing exciting energy causing its return to ground state oxygen, or react to produce tocopherylquinone (Smirnoff, 2005b). In plants, it has been recognized that the efficiency of tocopherols as antioxidants *in vitro* follows the order $\delta > \beta = \gamma > \alpha$. Tocotrienols can have equal or greater antioxidant capacity than tocopherols (Rippert et al., 2004). In algae, tocopherols have been observed to quench $^1O_2$ in photosystem II of the unicellular green alga *Chlamydomonas reinhardtii* (Trebst et al., 2002). A transcriptomic study developed by Luis et al. (2006) found that genes involved in the synthesis of α-tocopherol in *C. reinhardtii* are over-expressed during periods of high Cu exposure, protecting the thylakoid membrane integrity from oxidative stress. In addition, observations on an α-tocopherol-lacking mutant of *C .reinhardtii*, with only β-tocopherol, survived better under photo-oxidative stress (Sirikhachornkit et al., 2009). The production of tocopherols and tocotrienols in response to ROS induced by exposure to environmental stressors such as ultraviolet exposure (Malanga and Puntarulo, 1995; Hernando et al., 2005), salinity (Jahnke and White, 2003), seasonality and temperature (Collen and Davison, 2001) have been documented in algae. However, no published information has been found in regard to vitamin E activity under metal-mediated oxidative stress. Research on this direction will help elucidate the importance of these isoprenoids in the overall defence machinery against metal stress in algae.

In addition to their metal chelating characteristics, phenolic compounds have also been found to be efficient antioxidants (Dai and Mumper, 2010; Ruzic et al., 2010). The major classes of phenolics are flavonoids, tannins, hydroxycinnamic acids (HCAs) and anthocyanins; these compounds have been classified as secondary metabolites,

because they are not directly involved in growth and development of living organisms (Croteau et al., 2000). The antioxidant capacity of phenolic compounds relates to the electron donating capacity of their acidic hydroxyl group. Oxygen radicals oxidise phenolic compounds to phenoxyl radicals, which are generally less reactive than ROS (Buettner, 1993; Bors et al., 1994; Pedrielli et al., 2001). In algae, the importance of phenolic compounds in counteracting metal-induced oxidative stress has been documented. For example, Kovacik et al. (2010) measured phenolic production under Cu stress in the green alga *Scenedesmus quadricauda*, and Connan and Stengel (2011) in the brown fucoids *Ascophyllum nodosum* and *Fucus vesiculosus*. Both studies observed a decrease in the levels of phenolics with increasing Cu exposure; however, it is important to point out that the levels of total Cu used were as high as 1.5 mg L^{-1} in case of *S. quadricauda,* and 5 mg L^{-1} for *A. nodosum* and *F. vesiculosus*. Thus, these results should be treated cautiously due the use of non-realistic environmental concentrations. The studies described were performed under levels of Cu that can rarely be observed in nature, even in highly polluted environments (e.g. Correa et al., 1999; Canning-Clode et al., 2011), suggesting that depletion of phenolic content could have been caused by saturation instead of inhibition.

An important part of the ROM of living organisms is the activity of antioxidant enzymes. It has been recognized that these enzymes catalyse reactions that reduce ROS to less reactive molecules. The enzyme superoxide dismutase (SOD) is the first line of defence against ROS, catalysing the dismutation of $\bullet O_2^-$ into O_2 and H_2O_2 (Ken et al., 2005); although H_2O_2 is a less harmful ROS compared to $\bullet O_2^-$**,** its over-production can also produce extensive cell damage (Apel and Hirt, 2004). The activity of different SOD iso-enzymes (Mn-SOD in mitochondria, Fe-SOD in chloroplasts, and Cu-Zn-SOD in

the cytosol) provides important protection against $\bullet O_2^-$ excess (Fridovich, 1997). For example, the activity of SOD has been observed to increase in the green microalga *Tetraselmis gracilis* (Okamoto et al., 1996) and in the red seaweed *Gracilaria tenuistipitata* (Collen et al., 2003) under chronic Cd and Cu stress, respectively. Although there is no published information on SOD under metal stress in brown algae, it has been found that its activity can increase under exposure to other oxidative stress inducers, as observed in *Fucus vesiculosus* under exposure to the cyclic pentapeptide nodularin (Pflugmacher et al., 2007), produced in cyanobaterial blooms. A comprehensive review of the role of SODs iso-enzymes in algae have been recently published by Wolfe-Simon et al. (2005).

In order to counteract the effects of high levels of H_2O_2, algae have developed a strong defence mechanism based on the activity of a battery of peroxidase enzymes, among which can be highlighted the enzymes ascorbate peroxidase (APx), catalase (CAT), and glutathione peroxidase (GPx). APx is only present in photosynthetic organisms, and catalyses electron transfer from ASC to H_2O_2, which results in the production of MDHA and H_2O (Asada, 1992); APx is an essential component of the glutathione-ascorbate cycle in organisms such as plants and algae (Noctor and Foyer, 1998). The activity of APx has been observed to increase in the green alga *U. compressa* (Ratkevicius et al., 2003) and in the intertidal brown seaweeds *Lessonia nigrescens* and *Scytosiphon lomentaria* in highly Cu polluted environments relative to pristine locations (Contreras et al., 2009). CAT is one of the most common antioxidant enzymes in living organisms, catalysing the decomposition of H_2O_2 into H_2O and O_2 (Aebi, 1984). CAT activity has been recorded in several algal species under metal stress; for instance, Pereira et al. (2009) found higher CAT activity in *Ulva sp.* during

autumn and spring in Barrosa Branch, Portugal, which was concomitant with the increase in the levels of Cu and Ni in these seasons. Moreover, Wu and Lee (2008) measured the expression of CAT gene and its activity in *Ulva fasciata* under Cu exposure ranging between 0 and 50 µM and observed upregulation of gene expression and higher CAT activity with increasing Cu concentrations. The enzyme GPx catalyses electron transfer from GSH to H_2O_2, producing GSSG and H_2O (Noctor et al., 2012). Although this enzyme has been mainly studied in microalgae, its activity buffers against metal-mediated H_2O_2 production. For example, Rai et al. (2013) found an increase in the activity of GPx in the green alga *Chlorella vulgaris* under increasing exposure to Cr between 0 and 1.6 µM. On the other hand, Li et al. (2006) observed that GPx activity in the microalga *Pavlova viridis* increased under Cu exposure but decreased under Zn stress; however, levels of exposure were environmentally unrealistic, 3 mg L^{-1} and 6.5 mg L^{-1} for Cu and Zn, respectively. From the available published information on the activity of peroxidase enzymes, it appears that their role in the antioxidant machinery is species-specific and highly dependent on variables such as the metal itself and the levels of exposure. Moreover, it has been found that different peroxidases can cooperate in their action against H_2O_2; for instance, even though Contreras et al. (2009) found that APx and CAT activities both increased in the brown seaweeds *Lessonia nigrescens* and *Scytosiphon lomentaria* under Cu exposure up to 1.6 µM, their activities were much higher in *L. nigrescens* than in *S. lomentaria*.

Several other enzymes that do not directly interact with ROS and, therefore, are not strictly antioxidant enzymes, play an important role in the glutathione-ascorbate cycle. The enzyme glutathione reductase (GR) catalyses the reduction of GSSG back to GSH, using one mole of NADPH (Noctor et al., 2012); this reaction is essential in order

to recycle GSH, which, as outlined above, is one of the most important cellular antioxidants). Huang et al. (2013) observed that the activity of GR in the red seaweed *Gracilaria lemaneiformis* was lower than in *Gracilaria lichenoides* under increasing levels of free Cu (0-500 µg L^{-1} Cu^{2+}), which was coincident with higher concentrations of ROS and lipoperoxides in *G. lemaneiformis* relative to *G. lichenoides.* In a seasonal study, Pereira et al. (2009) observed that the activity of GR was greater in *Ulva sp.* sampled during autumn in Barrosa branch, Portugal, which corresponded with an increase in the seawater content of Cu and Ni. The information suggests that the activity of GR increases in order to have an efficient GSH recycling to counteract metal-mediated oxidative stress. Another important enzyme within the glutathione-ascorbate cycle is monodehydroascorbate reductase (MDHAR), which catalyses the reaction between MDHA, NADH, and H^+, allowing recycling of ASC (Noctor and Foyer, 1998). Although MDHAR has been identified and measured in cyanobacteria (Obinger et al., 1998) and green microalgae (Haghjou et al., 2009) under different environmental stressors, no data could be found on the activity of this enzyme in algae under metal stress. However, information is available on vascular plants. For example, Karuppanapandian and Kim (2013) observed an increase in the activity of MDHAR in the vascular plant *Brassica juncea* when exposed to 100 µM Co and Lyubenova and Schroder (2011) found similar patterns of activity in *Typha latifolia* under Cd, As, and Pb stress. In the latter example, the pattern of increase was observed in leaves but not rhizomes. When ascorbate is oxidised to DHA, the enzyme dehydroascrobate reductase (DHAR) is responsible for catalysing the reaction between two moles of GSH and one mole of DHA, producing one molecule each of GSSG and ASC; this is another important pathway for the recycling of ASC (Noctor and Foyer, 1998). Contreras et al. (2009) found that in the two kelp species *L. nigrescens* and *S.*

lomentaria the activity of DHAR increased with Cu exposure of up to 1.6 µM; DHAR activity was usually higher in *S. lomentaria.*

1.5 *Ectocarpus siliculosus*

Ectocarpus siliculosus is a small filamentous brown alga belonging to the order Ectocarpales (Figure 1.1). It is closely related to more complex seaweeds such as Laminariales and Fucales (see Figure1.2), key structuring components of coastal ecosystems in temperate environments (Smith, 1996b; Smith et al., 1996; Villegas et al., 2008; Sáez et al., 2012b). *Ectocarpus* is distributed worldwide in temperate regions, especially in marine and low salinity habitats; however, it has also been found in freshwater (West and Kraft, 1996) and hyper-saline (Geissler, 1983) ecosystems. This species occur from the high intertidal to the subtidal, attached to abiotic substrata (fouling alga) or as an epiphyte of more complex seaweeds (Russell, 1976; Russell, 1983a).

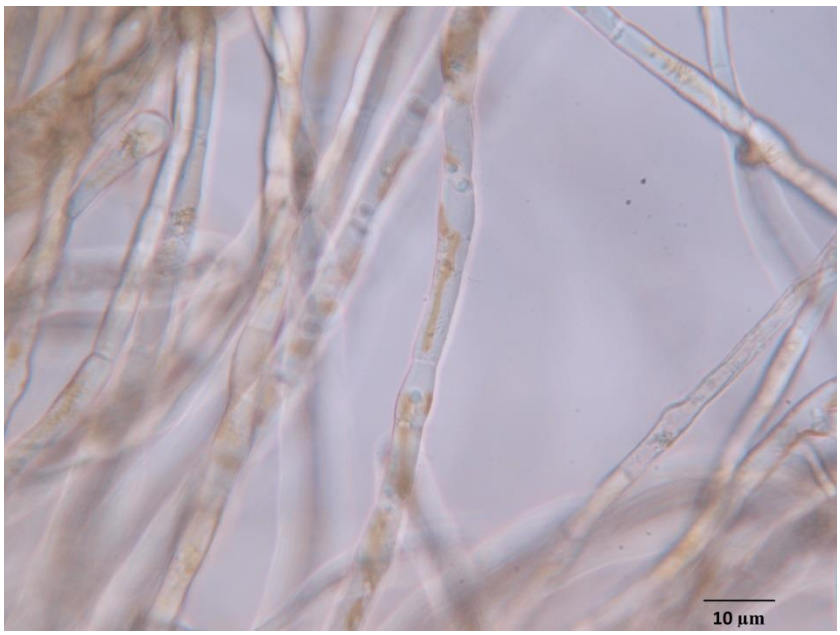

Figure 1.1. *Ectocarpus siliculosus*

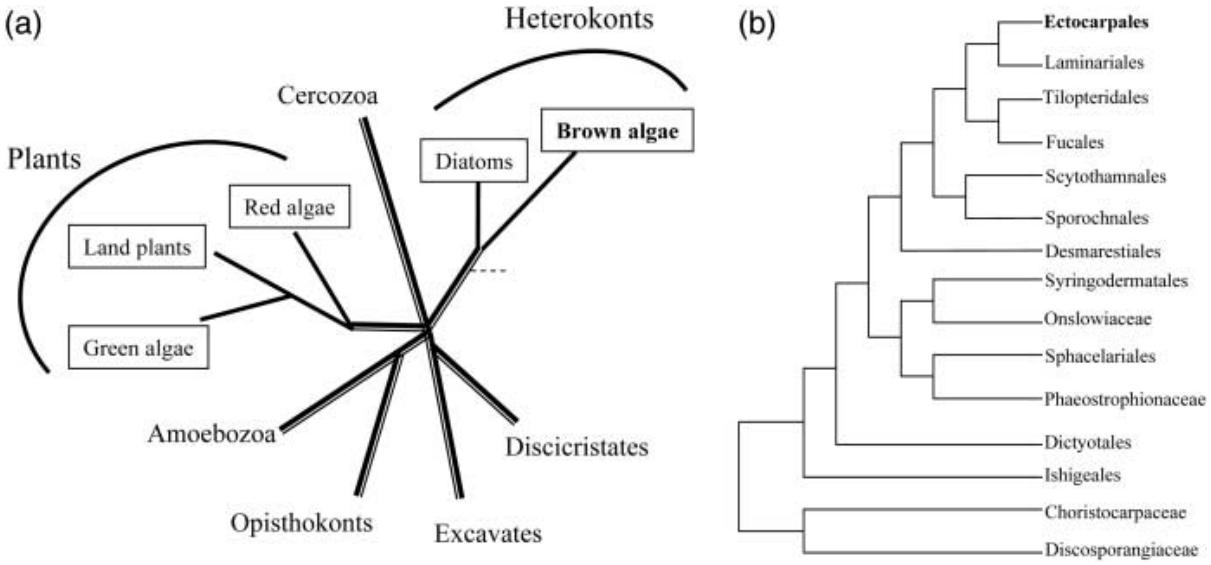

Figure 1.2. Phylogeny of brown algae and Ectocarpales (from Charrier et al., 2008). (a) Brown algae within eukaryotes. (b) Ectocarpales (bold) within the brown algae.

Research on *E. siliculosus* is extensive in different topics (see Charrier et al., 2008), starting on the 19th century with its description and taxonomic designation

(Dillwyn, 1809). Later on, research has been carried out on its life cycle, metabolism, biochemistry, stress resistance, viruses, and genetics, among others (Coelho et al., 2012b). Recently, Peters et al. (2004a) proposed *E. siliculosus* as a general model organism for the brown algae. The latter was first based on the long research history behind this species, but also in many other aspects that make *E. siliculosus* a potential good model organism, for example, its small size and that the complete life cycle can be completed in Petri dishes in the laboratory (Müller et al., 1998), fast growth, ease to perform genetic crosses, and a relative small genome size of 214 Mbp (Cock et al., 2010). The recent publication of the genome by Cock et al. (2010) has opened the window for new research that will help clarifying several unknown aspects of *E. siliculosus* biology and, more generally, of brown algae. Species belonging to the genus *Ectocarpus* can have high genetic differentiation and important variability in metabolic and physiological aspects. Microarray analyses of EST sequences from the available genome (reference strain CCAP 1310/4) have demonstrated high genomic differentiation between strains of *E. siliculosus*, supporting the hypothesis of the existence of cryptic species still to be discovered (Dittami, Simon M. et al., 2011).

Ectocarpus siliculosus has a haploid-diploid cycle, which leads to the production of different generations of sporophytes (2n) and gametophytes (n) (Bothwell et al., 2010). Sporophytes are able to produce meiospores in unilocular sporangia, which can develop into male or female haploid gametophytes. On the other hand, gametophytes produce diploid gametes in plurilocular gametangia; they can form a zygote to grow into a new diploid sporophyte and complete the cycle. The latter is just a simple example of alife cycle completion, there are several exceptions and different pathways, recently reviewed in depth by Charrier et al. (2008).

After the germination of the zygote, division starts with two identical cells (Peters et al., 2004b). Further mitosis forms filaments within ca.10 days made of round cells at the middle and elongated cells at both ends. After ca. 5 days secondary filaments start to grow forming what is called a prostrate body (with similar function to holdfast in kelps and fucoids), from which upright filaments branch off in order to develop an adult sporophyte (Le Bail et al., 2008a). In contrast, gametophytes tend to have an asymmetrical mitotic division from the zygote, producing a structure with less developed prostrate body; thus it has been observed that gametophytes tend to grow detached from a hard surface when cultured in the laboratory (Bothwell et al., 2010).

Information about the ecology of *Ectocarpus* is restricted as the majority of research has been carried out in the laboratory. Field studies are difficult with *Ectocarpus* mainly for its size and for several features that make different species difficult to distinguish, such as habits, filament patterns, size of reproductive organs, recognition of diploid or haploid stages, among others (Charrier et al., 2008).

It is known that growth of *Ectocarpus* is dependent on temperature, which has been observed to influence life cycles in some strains (Bolton, 1983). Recent observations on *Ectocarpus crouaniorum* in Brittany, France, have demonstrated that epilithic sporophytes are small and occur throughout the year, while gametophytes are ephemeral, only found during spring (Peters et al., 2010). Therefore, it might be expected that the sporophyte generation is more adapted to environmental changes.

Ectocarpus can be found in different zones of the intertidal and subtidal. In Brittany, it has been observed that *E. crouaniorum* occurs in the upper zone of the intertidal, while *E. siliculosus* and *E. fasciculatus* are distributed in the middle intertidal and subtidal, respectively (Peters et al., 2010). They can occur as epiphytes, for example as endophytes in blades of *Laminaria digitata* (Russell, 1983b), or epilithically (Peters et al., 2010). *Ectocarpus* can be also found as a floating thallus, which has been shown to be usually morphologically different from attached individuals (Russell, 1967a; Russell, 1967b). It is known that the *Ectocarpus* thalli provide habitat to a range of small biota, although their interactions remain not clear (Coelho et al., 2012b).

1.6 Problem and aims of the project

Stress biology and ecotoxicology research related to brown algae under metal stress is extensive, specially due to their sessile nature, high accumulation capacity, and also due to the metal resistance of some of their species (Sáez et al., 2012a). It has been observed that in addition to the inter-specific responses to metal stress observed in brown algae, they can also develop intra-specific adaptations after a long history of metal exposure, allowing them to develop tolerant populations that can thrive in highly metal-polluted environments (Pawlik-Skowronska et al., 2007; Ritter et al., 2010). However, there is a lack of information regarding the mechanistic basis of inter-population response to metals. Now with sufficient information about *E. siliculosus* biology, the genome available, and a collection of strains with origin in different polluted and pristine locations, there is an unprecedented opportunity to further study intra-specific adaptations to metal stress in brown algae.

The aim of our investigation was to assess the basis of inter-population resistance in different strains of *E. siliculosus* under Cu stress at different levels of biological organization, through a combination of laboratory and field studies. In the Chapter II of this thesis we provide a description of general materials and methods used for *E. siliculosus* culturing and manipulation. In Chapter III we describe our findings on growth, Cu accumulation (extra- and intra-cellular), and the levels of total glutathione and PCs, and the expression of related enzymes, in two strains of *E. siliculosus* exposed in the laboratory to Cu concentrations ranging from 0 to 2.4 µM for 10 days; the strains used were the Es524 (CCAP 1310/4) collected in a Cu polluted location of northern Chile, and the strain LIA4A, sampled in a pristine location in Scotland. Under the same experimental conditions as those used in Chapter III, in Chapter IV we present our results on several observations made on the reactive oxygen metabolism (ROM) of different *E. siliculosus* strains; in addition to Es524 and LIA4A we incorporated the strain REP10-11, originated in a metal polluted (also Cu) location of south-west England. We measured levels of lipid peroxidation, hydrogen peroxide, and the content of chlorophylls *a* and *b*, and fucoxanthin, the content of glutathione and ascorbate in reduced and oxidised forms, phenolic compounds, and the activity of the antioxidant enzymes SOD, CAT and APx. In order to ascertain our findings in the laboratory, in Chapter V we conducted novel *in situ* transplantation experiments in a metal polluted (including Cu) and pristine locations of central Chile, and conducted similar measurements of the ROM as described in Chapter IV; For this, we used the strains Es524 and Es147, the latter from a low metal polluted location of northern Chile.

After conducting gene expression analyses in Chapter III, we observed that the available published records for RNA extraction were not completely successful to

obtain good yields of high quality RNA from different strains of *E. siliculosus*. To overcome current issues related to poor nucleic acids-extraction using the available methods, we developed a new method for high quality co-extraction of RNA and DNA, which permitted obtaining high yields of pure nucleic acids of different strains of *E. siliculosus*. In Chapter VI we provide a detailed description of the protocol developed; we validated our protocol by assessing the quality of the extracted nucleic acids through different downstream applications.

In Chapter VII we provide a general discussion of the different aspects covered in the thesis and discuss their overall importance to further understand metal stress metabolism in *E. siliculosus* and brown algae and, furthermore, their implications for biomonitoring using brown seaweeds oxidative stress biomarkers. Moreover, we describe future work, which arose from the findings obtained during the course of this investigation.

Chapter II

General materials and methods

2.1. *Ectocarpus siliculosus* manipulation

Methods courtesy of Sation Biologique of Roscoff, France.

2.1.1. Preparation of laminar flow hood

The hood was cleaned with ethanol (70% ethanol in distilled water) before manipulation of cultures. After switching the hood it was left running for 10 min before using it. Absorbent paper was used to clean the hood during the manipulation. Necessary materials such as culture medium, Provasoli nutrients, tweezers, parafilm, ethanol, pipettes, bunsen, and matches were kept inside the hood. Hands and tweezers were washed regularly with ethanol to reduce risk of biological contamination.

2.1.2. Manipulation of cultures

Hands were cleaned with ethanol before starting, after handling Petri dishes, between strains and after having taken something from outside the hood. Tweezers were washed in pure ethanol after working with Petri dishes or in between strains. While working, all plastic ware waste (dishes, polycarbonate flasks, pipette tips, blue paper tissue, multi-well plates, petri dishes, etc.) were disposed in autoclavable plastic bags.

2.1.3. Maintenance of cultures

Freshly made media, consisting in autoclaved natural seawater (NSW) plus Provasoli nutrients (media preparation described later in this Chapter), was replaced every 1 or 3 weeks to stimulate growth. The latter should be done in a horizontal laminar flow hood as described in the past section. Fluorescent light has to be provided at 20-30 µmol

m^{-2} s^{-1} photon irradiance rate, 15°C, with regimes of 14/10 hours of light/dark, respectively.

To produce more biomass, bottles of 10 L were used (Figure 2.1). In this case, aeration through a 0.22µm filter was provided to avoid CO_2 depletion.

Figure 2.1. *Ectocarpus siliculosus* cultures in 10 L bottles.

2.2. Culture and experimental media preparation

2.2.1. Provasoli enrichment for natural seawater

All glass-ware and plastic-ware used throughout this research was washed in HCl (5%) for at least 24 hours, in order to clean material and remove any adsorbed metals, then rinsed thoroughly with milli-Q water.

The algal material was cultured and maintained in Provasoli-enriched natural seawater (Provasoli and Carlucci, 1974) using a modified protocol provided by the Station Biologique Roscoff, France (Table 2.1). All stock solutions were prepared separately, autoclaved at 122°C for at least 30 min, and later stored at 4°C in glass amber bottles.

Table 2.1. Solutions for preparation of Provasoli

Solution 1	**Concentrations**	**g L^{-1}**
H_3BO_3	30.7 mM	1.9
$FeCl_3$	0.3 mM	0.05
$MnSO_4$ (H_2O)	1.6 mM	0.273
$ZnSO_4$ (7 H_2O)	0.127 mM	0.0367
$CoSO_4$ (7 H_2O)	28 µM	0.008
EDTA	5.7 mM	1.67 mL L^{-1}
Solution 2		
Vitamine B_{12} (cyanocobalamine)	0.0067 g/liter	0.00335
Thiamine hydrochloride (vitamine B1)	0.33	0.165
Biotine C10H16N2O3S	0.0033	0.00165
TRIS = Tryma base C_4H_{11} NO_3	333.0	166.5
Solution 3		
$(NH_4)2Fe(SO_4)2(6H_2O)$	3mM	1.17
EDTA 0.5 M pH8	3.4mM	6.8 mL L^{-1}
Solution 4		
$NaNO_3$	270 mM	23
Solution 5		
5 $C_3H_7Na_2O_6P$ ($5H_2O$)	15.4 mM	3.33

To prepare Provasoli medium, the five solutions described before were mixed. To prepare 1 L, 100 mL of each one of the solutions was added, excepting solution 2 (10 mL). The volume then was increased up to 1 L with Milli-Q; at this stage the pH was between 9.6 and 9.8. The pH was adjusted to 7.8 with 0.1 M HCl solution. To avoid contamination by over-manipulation, Provasoli medium was stored in small glass bottles (20, 50, 100, 200 mL). Provasoli was autoclaved and then stored at 4°C. The use of Provasoli medium was 10 mL per litter of NSW.

2.2.2. Artificial seawater for metal experiments (Aquil)

For experimentation, *E. siliculosus* was grown in a modified version of the chemically defined seawater medium Aquil (Morel et al., 1979; Price et al., 1989). Ethylenediaminetetraacetic acid (EDTA) was eliminated from the recipe to prevent metal chelation. Aquil was originally developed for studies of trace metals in planktonic algae and has been successfully applied to studies of brown macroalgae (e.g. Bond et al., 1999; Gledhill et al., 1999; Nielsen et al., 2003; Nielsen and Nielsen, 2010). Using Aquil allows the bioavailable fraction of the total concentration of metal added to be calculated using the chemical equilibrium program Windermere Humic Aqueous Model [WHAM] (Nielsen et al., 2003).

Aquil was prepared in three parts: synthetic ocean water (SOW), nutrients, and trace metals (Table 2.2). Filtered sterilised stock solutions were prepared using milli-Q water (18 Ω). Nutrients and trace metals were added to SOW.

2.1.2.1. Vessels treatment, sterilization and storage

Once prepared, Aquil media and nutrient stocks solutions were kept in polycarbonate and polyethylene bottles which are largely free from trace metals and adsorb only small amount of metals. Exception for phosphate nutrient stock solution which was kept in glass bottles as PO_4 strongly adsorbs to polyethylene. Preparation of medium was done under a laminar flow hood.

Synthetic Ocean Water (SOW) were autoclaved; nutrient and trace metals stock solutions were filtered, sterilized with 0.22 µm filters to reduce biological contamination and precipitation during autoclave sterilizing procedure. In addition, trace metals stock solutions were prepared in 0.01 M HCl in order to avoid loss of trace metals due to precipitation.

Nutrient and trace metal stock solutions were kept in dark at 4 degrees for up to three months. Medium was kept at 4°C if not needed or in cabinet at the temperature of algal growth (15°C). Nutrients and trace metal stock solutions were prepared individually final concentrations and then added to SOW.

2.2.2.2. SOW preparation

To obtain SOW (Solution 1 in Table 2.2), first all anhydrated salts were dissolved in milli-Q water. After, hydrate salts were added and dissolved. Final pH was 8.2, which was brought to 8.1 with 0.1 M NaOH (in the autoclave it will rise to the final pH of 8.2). SOW was transferred into polycarbonate or Teflon bottles and autoclaved. When SOW cooled down, the nutrients and trace metals stock solutions were added under a laminar

hood. Bottle necks were flamed with a Bunsen burner under a laminar hood before closing them. Aquil was stored at 4°C if not immediately needed or in cabinet at the temperature required for algal growth. When Aquil was used for Cu exposure experiments, Cu was added from a concentrated stock solution (filtered and sterilized with 0.22 µm filter, and kept in dark at 4°C). Cu needswas added to the medium the day before the experiment to allow for chemical stabilization (further details in Chapters III and IV of this thesis).

2.2.2.3. Aquil preparation

Each chemical solution within Solution 2 needs to be prepared separately, as described in Table 2.2. Then, 1 mL of each nutrient solution is added to 1 L of SOW, excepting solution KI, which is 332 µL in 1 L SOW.

Prepare trace metals solutions 1, 2, 3, and 4 need to be prepared separately as shown in Table 2.2. Then, 100 µL trace metal solutions 2, 3, and 4 need to be added to trace metal solution 1. Finally, 1 mL of new trace metal solution 1 are added to 1 L of SOW.

Table 2.2. Composition of synthetic ocean water (SOW)

Solution 1: SOW

Anhydrated salts	**g L^{-1}**	**Concentration (M)**
NaCl	24.03	0.42
Na_2SO_4	4.09	0.0288
KBr	0.1	0.00084
KCl	0.7	0.00939
H_3BO_3	0.03	0.000485
$NaHCO_3$	0.02	0.00238
Hydrated salts		
$CaCl_2\,2H_2O$	1.51	0.0105
$MgCl_2\,6H_2O$	11.1	0.0546
Solution 2: Nutrients	**Grams per 100 mL of 0.01 M HCl**	**Concentration (M)**
$SrCl_2\,6H_2O$	0.0638	$6.38 * 10^{-5}$
NaF	0.0714	$7.14 * 10^{-5}$
KI	0.000723	$2.4 * 10^{-7}$
$NaNO_3$	0.24	$2.4 * 10^{-4}$
NaH_2PO_4	0.015	$1.5 * 10^{-5}$
Solution 3: Trace metal 1	**Grams per 100 mL of 0.01 M HCl**	**Concentration (M)**
$FeCl_3\,6H_2O$	0.0122	$4.51 * 10^{-4}$
Solution 4: Trace metal 2	**Grams per 100 mL of 0.01 M HCl**	**Concentration (M)**
$Na_2MoO_4\,2H_2O$	2.42	0.1
$CoCl_2\,6H_2O$	0.0595	$2.50 * 10^{-3}$
Solution 5: Trace metal 3	**Grams per 100 mL of 0.01 M HCl**	**Concentration (M)**
$ZnSO_4\,7H_2O$	0.115	$4.0 * 10^{-3}$
$MnCl_2\,4H_2O$	0.46	$2.3 * 10^{-2}$
Solution 6: Trace metal 4	**Grams per 100 mL of 0.01 M HCl**	**Concentration (M)**
$CuSO_4\,5H_2O$	0.0249	$9.97 * 10^{-4}$

2.3. Methods for physiological, analytical, molecular, and biochemical analyses

To facilitate the understanding of the results and discussion sections of every chapter of this thesis, the specific methodologies applied regarding experimental design and analyses conducted were explained in detail in every chapter. Moreover, because some of the protocols were adjusted during the course of this project to improve the precision and accuracy of the results.

In Chapter III, we applied different measurements related to physiology, Cu accumulation, glutathione and phytochelatin levels, and related gene expression, in two *E. siliculosus* strains after Cu exposure. Relative growth rates were measured with the Wintrobe tube method first described by Dring (1967), and calculated according to Yong et al. (2013). Cell wall adsorbed and intracellular concentrations of total Cu accumulated were obtained after washing the biomass with an EDTA solution as described by Hassler et al. (2004), and measured by ICP-MS. Gene expression analyses were carried out after extracting RNA with modifications to the method stated in Apt et al. (1995). We studied the genes encoding the enzymes glutamate cysteine ligase, glutathione synthase (both in the pathway of GSH synthesis), and phytochelatin synthesis (catalyser of phytochelatin production from GSH); data were normalized to the expression of alpha tubulin, and levels of expression were calculated according to Livak and Schmittgen (2001). The concentrations of total glutathione and phytochelatins were measured after thiol extraction according to Knecht et al. (1994) and Pawlik-Skowrońska et al. (2007). After reduction of thiols, total glutathione and phytochelatins were determined through HPLC with modifications to the protocol published by Gledhill et al. (2012).

In Chapter IV, we measured different aspects of the reactive oxygen metabolism (ROM) of three strains of *E. siliculosus* under a range of Cu exposures. First, we measured

lipid peroxidation and hydrogen peroxide content according to Sergiev et al. (1997), and chlorophylls *a* and *c*, as well as fucoxanthin according to Seely et al. (1972). The concentrations of glutathione in reduced (GSH) and oxidized (GSSG) forms were measured according to Queval and Noctor (2007). For ascorbate in reduced (Asc) and oxidised (DHA) forms, extracts were prepared as for GSH and GSSG, and measured as described by Benzie and Strain (1999). The levels of total phenolic compounds were determined with the method by van Alstyne (1995). We also measured the activity of different antioxidant enzymes, after preparing protein extracts with the protocol described in Collen and Davison (1999); the activity of the antioxidant enzymes was expressed per grams of fresh weight biomass. We followed the activity of superoxide dismutase (SOD) according to McCord and Fridovich (1969), and calculated its activity as stated in Kuthan et al. (1986). Finally, the activities of catalase (CAT) and ascorbate peroxidise (APx) were measured and calculated according to Collen and Davison (1999).

In Chapter V, we performed transplantation experiments in metal (mainly Cu) polluted and pristine locations of central Chile, using two strains of *E. siliculosus*. The measurements conducted were the same as described for Chapter IV regarding the ROM; however, the main differences lie on how the extracts were prepared for determination of the activity of antioxidant enzymes. In this occasion, we prepared extracts as described in Ratkevicius et al. (2003), which uses ammonium sulphate to precipitate and concentrate proteins; we changed the procedure because with the former protocol modified from Collen and Davison (1999), the levels of proteins were low and that represented difficulties when measuring the activity of antioxidant enzymes. Moreover, with the new extraction protocol we were able to measure the activity of another antioxidant enzyme; in addition to SOD, CAT, and APx, we could determine the activity of glutathione reductase (GR).

After conducting RNA extraction from different strains of *E. siliculosus*, as presented in Chapter III for gene expression analyses, we observed that the yields and quality of the RNA were different for the strains used, and therefore the extraction procedure adapted from Apt et al. (1995) needed different steps of purificationdepending on the strain. Moreover, the procedure required at least 100 mg of biomass, which is high taking into account the low biomass production of *E. siliculosus*. In addition, later on we observed that the method by Le Bail et al. (2008b) for total RNA presented similar issues as mentioned for the protocol by Apt et al. (1995). To overcome the latter issues, in Chapter VI we describe a new designed method which proved to be successful for the co-extraction of high yields of pure RNA and DNA from low biomass (down to 25 mg of biomass) of different strains of *E. siliculosus*.

Different statistical analyses were performed throughout this thesis, which were mostly parametric. When possible, statistical indicators where displayed in figures and tables to provide more information for the reader. Nevertheless, when visual representation of statistical differences was not possible, for example if performing a multifactor ANOVA, the detailed information was rather presented in text format in results sections.

References were formatted as for the journal New Phytologist, excluding information about the specific issue in case of journal articles.

Chapter III

Intra-specific variation in glutathione-phytochelatin production and expression of related enzymes in *Ectocarpus siliculosus*

3.1. Introduction

Ectocarpus siliculosus is a cosmopolitan filamentous brown alga that can live in a range of habitats and environmental conditions, from fully marine to low-salinity (Charrier et al., 2008). Ectocarpoids are a group of bio-fouling organisms, that grow on different substrata, both biotic (as epiphytes on macrophytes) and abiotic (rock, ship hulls, wood, plastic) and also as free-floating mats (Russell, 1967a; Russell, 1967b; Russell, 1983a; Russell, 1983b). *Ectocarpus siliculosus* has been recently proposed as a model organism for the study of brown algae due to several important biological features including: small stature, ease of culture in the laboratory, short generation time, relatively small genome size (Peters et al., 2004a). The publication of the inventory of genes, following the sequencing of the entire genome and its annotation (Cock et al., 2010), has opened exciting new opportunities for post-genomic investigations, including furthering our understanding of the underlying mechanisms by which brown seaweeds respond to environmental stressors.

Copper (Cu) is an essential micronutrient for photosynthetic organisms, acting as cofactor of many enzymatic complexes and participating in crucial cellular processes such as signalling and photosynthesis (Brown et al., 2012). However, at high concentrations Cu can become toxic and affect these metabolic processes (e.g. Nagalakshmi and Prasad, 1998; Mellado et al., 2012). Resistance to metals might depend on mechanisms that involve both metal exclusion and intracellular defences. Exclusion mechanisms in algae can be related to different factors, such as the permeability of the cellular membrane, adsorption to the cell wall or epibionts (e.g. epiphytic bacteria), and the exudation of organic substances (e.g. polysaccharides, phenolic compounds) (Hall, 1981). On the other hand, the intracellular production of chelating agents to bind metals and the synthesis of low and high molecular weight antioxidants and activities of antioxidant enzymes to

prevent (or at least reduce) metal-induced oxidative damage are important components of the cell's response to metal stress (Pinto et al., 2003; Torres et al., 2008). Glutathione, a low molecular weight thiol, is one of the most important cellular antioxidants and metal chelators. In the presence of reactive oxygen species (ROS), reduced glutathione (GSH) is oxidised to glutathione disulphide (GSSG), which is then reduced back to GSH in a reaction catalysed by glutathione reductase (GR) (Noctor and Foyer, 1998; Noctor et al., 2012). GSH is also the primary pre-cursor of the intracellular metal-chelator phytochelatin (PCs) (Cobbett, 2000). PCs are small cystein- rich oligopetides (2 to 11 amino acids long) that chelate metals through their sulphydryl groups (Cobbett, 2000; Torres et al., 2008). PCs are synthesised through two enzymatic pathways: a) the synthesis of GSH by two consecutive ATP-dependent reactions involving γ-glutamylcysteine synthetase (GCL) and glutathione synthetase (GS), and b) polymerization of GSH to PCs by phytochelatin synthase (PCS) (Cobbett, 2000). Therefore, maintaining the equilibrium between synthesis and utilization of GSH and production of PCs is critical to counteract metal stress.

PC induction has been observed in different groups of photosynthetic organisms in response to high concentrations of metals and in particular to Cd (Cobbett, 2000). Most research has been focussed on higher plant taxa, including the model plant species *Arabidopsis thaliana* (Lee and Kang, 2005) and on microalgae (Scarano and Morelli, 2002; Kobayashi et al., 2006). In contrast, there is only limited evidence for metal-induced PCs synthesis in seaweeds; two recent investigations include those of Mellado et al (2012) on the green alga *Ulva compressa*, and Pawlik-Skowronska (2007) on several green (*Rhizoclonium tortuosum* and *Codium fragile*), red (*Solieria chordalis*) and brown (*Fucus* spp.) seaweeds.

It is known that in some plant and algal species a long history of metal exposure can mediate intra-specific responses to metal-stress, with enhanced metal resistance in populations growing in metal-contaminated environments and which is inherited to future generations (e.g. Ritter et al., 2010; Brown et al., 2012), whereas other species show innate resistance (Brown et al., 2012; Kirkey et al., 2012). However, while it has been recognized that GSH and PCs play important roles in metal detoxification and metal homeostasis (Cd mainly), their roles in differential metal resistance within species remain unclear, and furthermore in relation to other metals such as Zn and Cu. The aims of this investigation were to assess the differential responses in Cu-stress metabolism in two strains of the model brown alga *E. siliculosus*, one isolated from a metal-contaminated site and the other from a pristine location, from measurements of relative growth rates intra- and extra-cellular bound Cu, concentrations of total glutathione (GSH+GSSG) and PCs, and the transcriptional expression of *GCL*, *GS*, and *PCS* involved in GSH-PCs synthesis.

3.2. Material and methods

3.2.1. *Ectocarpus siliculosus* strains, culture conditions and Cu exposure

Samples from two strains of *E. siliculosus*, originally from locations with different histories of Cu exposure were used for the experiments: LIA4A, isolated from a pristine site in Scotland (Lon Liath), and Es524 (CCAP 1310/4) isolated from a Cu polluted site in Chile (Palito la Boca, Chanaral).

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 (18Ω). Strains were grown in 2 L polycarbonate bottles containing continuously aerated natural seawater enriched

with Provasoli nutrients (Provasoli and Carlucci, 1974), at 15 ^{0}C, 45 µmol photons m^{2} sec^{-1}, on a 14:10 light/dark cycle. When sufficient biomass had been produced, cultures were transferred to the chemically defined sea water medium Aquil (Morel et al., 1979), but without addition of a chelating agent the EDTA (Gledhill et al., 1999). Prior to experimentation, cultures were acclimatized for 10 d in Aquil under the same environmental conditions as described above.

Following the acclimatization period, strains were exposed in triplicates to a range of Cu treatments: control (no added Cu), 0.4, 0.8, 1.6, and 2.4 µM of nominal Cu. The free Cu (Cu^{2+}) concentrations present in the culture medium were calculated using the Windermere Humic Aqueous Model (WHAM7) software based on the Debye Huckel equation, employing a temperature of 288 K, pH of 8.2 and atmospheric pCO_2 (3.5×10^{-4} atm) (Nielsen et al., 2003). The equivalent free Cu concentrations were: 0.05 (control), 21, 42, 85, and 128 nM, respectively. The period of exposure was 10 d and fresh medium was added every 3 d.

3.2.2. Growth

The effect of Cu on growth of the *E. siliculosus* strains 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, and exposed to Cu concentrations mentioned above. The medium was replaced every 3 days. Volume variations (mm^{3}) were recorded at time of inoculation (t_0) and after 20 days (t_1). Growth rates were calculated using the formula $[(V_{t0}/V_{t1})^{1/t}-1]100\%$ (% d^{-1}) (Yong et al., 2013).

3.2.3. Metal analyses

Upon termination of the experiment, fresh biomass (FW) of 40 mg was taken from each replicate; excess water was removed by a few seconds of gentle vacuuming, and immediately frozen at -80^{0}C for the determination of total Cu accumulation analyses. To distinguish between total and intracellular (non-exchangeable) concentrations of accumulated Cu, a further 40 mg FW were washed twice (15 minutes each) in Aquil, containing 10 mM EDTA, in order to remove the exchangeable fraction of Cu bound to cell walls (Hassler et al., 2004), and then immediately frozen at -80^{0}C. Frozen biomass for total and intracellular Cu analysis were freeze-dried for 24 h, and then digested in a microwave (MARSXpress; cycle of 34 minutes at 120-170 ^{0}C), using 2 mL of 70% w/v HNO_3. Digested samples were diluted to 5 mL with milli-Q water and Cu content was determined by ICP-MS (Thermo Scientific, Hemel Hempstead, UK). Cu standards were made using certified solutions (ICP-AES standards obtained from Fisher Scientific) and internal standards (Yttrium and Indium) were also used.

3.2.4.1 Total RNA extraction and reverse transcription

Total RNA extraction was carried out, with modifications, using the method of Apt et al. (1995). All solutions were prepared with diethylpyrocarbonate (DEPC) water, to inactivate RNAse. 100 mg FW biomass 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). 1% β-mercaptoethanol was added to the buffer just 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 g for 20 min at 4^{0}C, the upper phase was transferred to a new tube, mixed with 0.2-0.3 V of absolute ethanol and 1 V of chlorophorm, 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 3M sodium acetate (pH 5.2) and 0.6 V of ice-cold isopropanol. The pellet was recovered by centrifugation at 21,000 g for 20 min at 4^0C, then washed with 75% ethanol, air dried and re-suspended in DEPC water. The RNA was treated with 30 U of I recombinant DNase (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 were 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.

3.2.4.2 Primer design

Gene sequences for the enzymes GCL, GS, and 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 GCL (*GCL1*, CBJ30287.1; *GCL2*, CBN79467.1) were studied (Table 3.1).

Table 3.1. 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/).

Gene name	Gene symbol	Gene ID	Accession number	Oligonucleotides – Forward - Reverse	PCR product size (bp)
Glutamate cysteine ligase	*GCL1*	Esi0184_0033	CBJ30287.1	ACGACTCCATCAGCACCTTC CGACAAAGCCGAGTCTATCC	146
Glutamate cysteine ligase	*GCL2*	Esi0250_0012	CBN79467.1	TGTCACACCCCTGTGAAGAA AGGGTAGTAGTCGCCCTTCC	149
Glutathione synthase	*GS*	Esi0066_0082	CBN75075.1	GAGGCTGTTGGAGATGAACC CGGAAGTAAACCACCGCTAT	150
Phytochelatin synthase	*PCS*	Esi0399_0022	CBJ32985.1	GACACACACACCCTCGAAGA GCACGAGGTCTTTCTCCTTG	163

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 maximize 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 CT values within the reactions of a triplicate. The average efficiency of the primer pairs used ranged between 0.95 and 1.0. Alpha tubulin (*TUA*) was selected as housekeeping gene as suggested by Le Bail et al. (2008b) for chemical stress.

3.2.4.3. Amplification conditions and data analysis

The qRT-PCR reaction was performed with a *StepOne* Real-Time PCR Instrument (Applied Biosystems, Italy) in a 20 µL total volume containing: 10 µl 2x PowerSYBR Green PCR Master Mix (Applied Biosystems, Italy), 400 nM of each primer, and 30 ng cDNA. Reactions were performed in triplicates with the following cycles: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. To test primer specificity, melting curve analysis (from 60°C to 95°C with an increasing heat rate of 0.5°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).

3.2.4. Glutathione and phytochelatin concentrations

Biomass (200 mg FW) was frozen at -80^{0}C and subsequently the non-protein thiols were extracted following the protocols of De Knecht et al. (1994) and Pawlik-Skowrońska et al. (2007). Samples were exhaustively extracted ice-cold with 1 mL of 5% 5-sulphosalicylic acid (SSA), containing 5 mM diethylentriamonopentaacetic acid (DTPA). Cells were disrupted by sonication (Vibracell ultrasonication processor) for 1 min (cycles of 10 seconds each; output 300 W). The extract was centrifuged at 10,000 g for 20 min at 4^{0}C, and the supernatant then syringe-filtered (0.20 µm pore size cellulose membrane, Minisart RC15, Sartorius).

Total glutathione and PCs were determined with modifications to the protocol of Gledhill et al. (2012). Thiols (250 µl of supernatant) were reduced using 25 µl of 20 mM 2-carboxyethylphosphine hydrochloride (TCEP) and the extract buffered at pH 8.2 with 160 µl of 200 mM HEPES. After 5 min incubation, 10 µl of a 100mM sulphur-specific

fluorescent tag monobromobimane (MBrB) was added (MBrB in acetonitrile, Fisher Scientific) followed by 465 µl of the HEPES/DTPA (pH 8.2) solution. The derivatization 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%, Sigma) and the extract stored in brown vials in the dark at 4^0C to await analsysis by HPLC. Thiols were quantified by reverse-phase HPLC with a fluorescence detector at wavelengths of 380 nm excitation and 470 nm emissions. Separation of the thiols was carried out using a 150 x 21 mm C-18 HPLC column (Ascentis, Supelco) with a 3 µm particle size, and was achieved using a gradient programme of acetonitrile and 0.1% trifluoroacetic acid (TFA, Fluka). The flow rate used was 0.2 mL min^{-1}. PCs concentrations were derived from a calibration curve using reduced glutathione (GSH) (99% purity, Sigma). PCs and total glutathione concentrations are expressed per g dry weight (DW), estimated from fresh weight to dry weight ratios (regression equation - DW = 0.80FW-0.001, R^2=0.812; P<0.001) derived after drying replicate samples (n=5) overnight at 90^0C. 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 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 Es524 after exposure to 189 nM Cd^{2+} (see Appendix I). Exposure to Cd provided evidence for production of PCs, with an increase in the content of PC2, PC3, and PC4, relative to the controls (Figures 3.1 and 3.2).

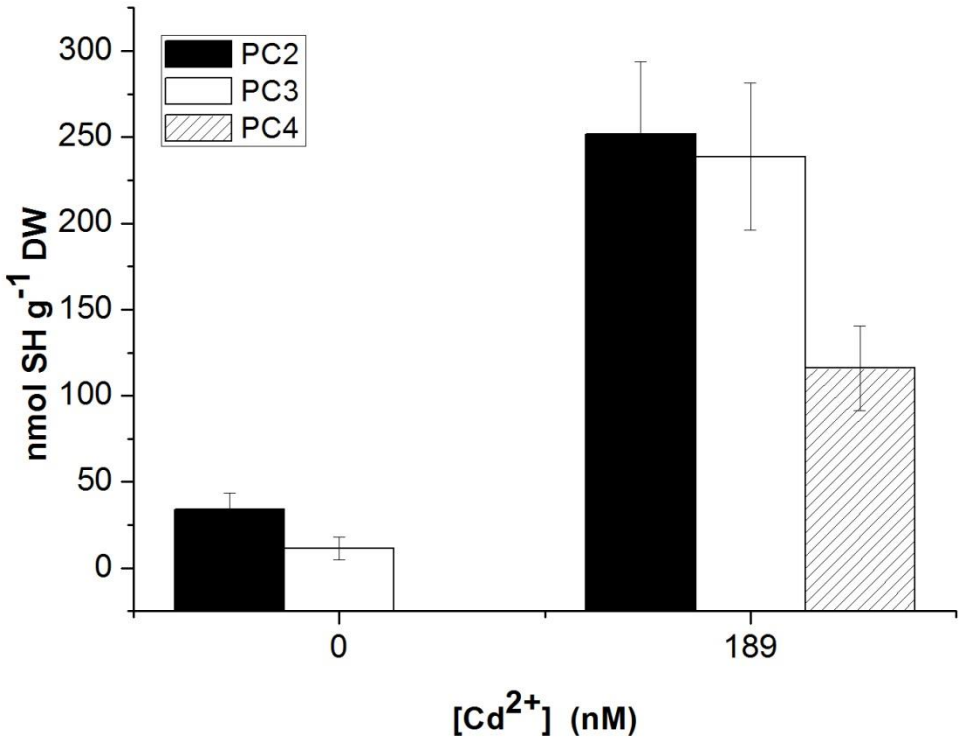

Figure 3.1. Concentrations of PCs in Es524 in control and exposed to 189 nM free Cd (Cd^{2+}). PC2, PC3 and PC4 dimers are shown. Error bars are ± 1 SD, n = 3.

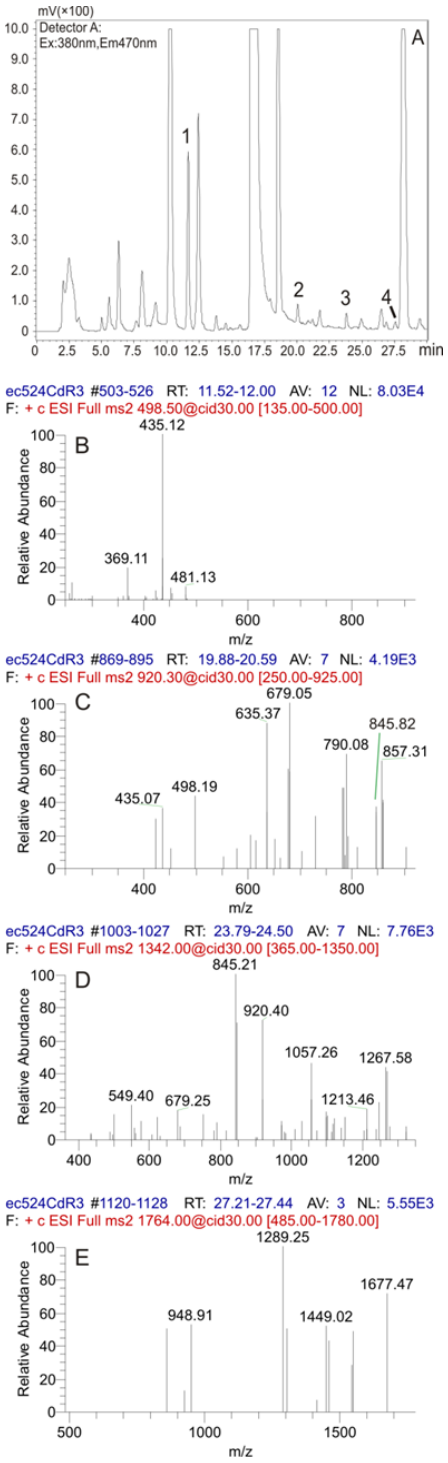

Figure 3.2 (A) Fluorescence chromatogram of extracts from Cd-exposed Es524. Peaks labelled 1-4 denote GSH, PC2, PC3 and PC4 respectively. (B-E) Average MS2 scans for GSH and PC2-4. RT – retention time, AV – number of scans averaged, NL – maximum ion count.

3.2.5. Statistical analysis

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) and post-hoc Tukey test at 95% confidence.

3.3. Results

3.3.1. Cu exposure and growth

For both strains the RGRs decreased with increasing Cu concentrations levels (one-way ANOVA, $p < 0.05$); RGRs were significantly lower for Es524 under control conditions and at 0.4 µMCu. Es524 showed significant growth reduction above 0.8 µM Cu ($p < 0.05$), while in LIA4A strain growth impairment was already apparent at 0.4 µM Cu ($p < 0.05$) (Figure 3.3); however, the pattern for the latter strain was interrupted at 1.6 µM Cu, with significantly higher RGR than at 0.8 µM Cu ($p < 0.05$).

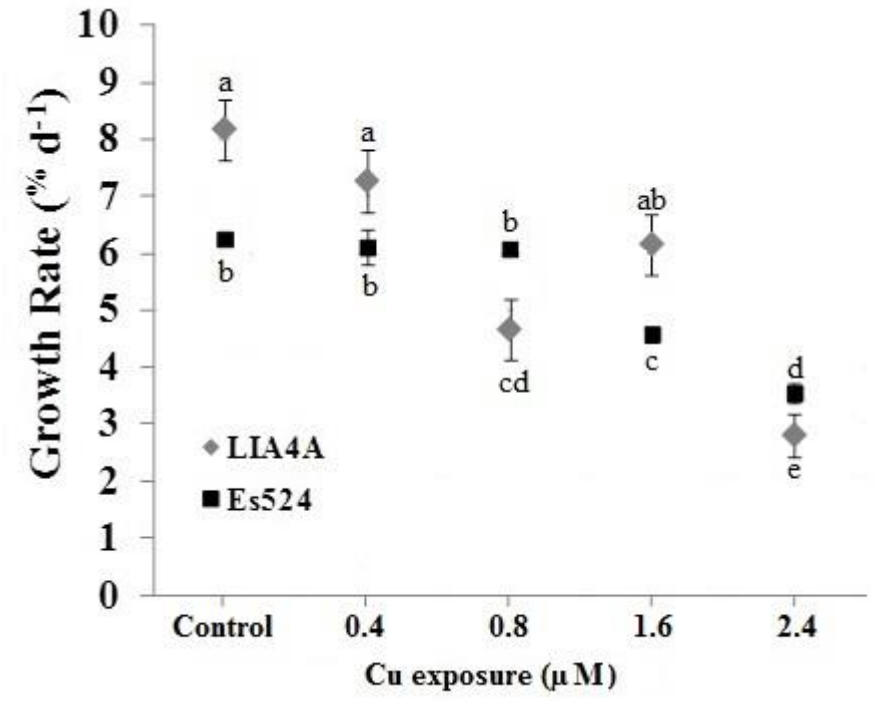

Figure 3.3. Relative growth rates of *E. siliculosus* strains Es524 and LIA4A exposed to Cu for 20 d. Relative Growth Rate is expressed as % d^{-1}. Es524 was originally collected from a highly Cu polluted site in Chile, while LIA4A was from a pristine site in Scotland. Error bars are ± 1 SD, n = 3.

3.3.2. Cu accumulation

The concentrations of intracellular and extracellular bound Cu were evaluated in control and Cu-exposed strains (Figure 3.4). In both LIA4A and Es524 strains the total (intra- + extra-cellular) Cu amount increased in response to metal exposure (Figure 3.4) (two-way ANOVA, $p < 0.05$); although at 0.8 and 1.6 µM Cu the accumulation levels were higher in Es524, compared to LIA4A ($p < 0.05$).

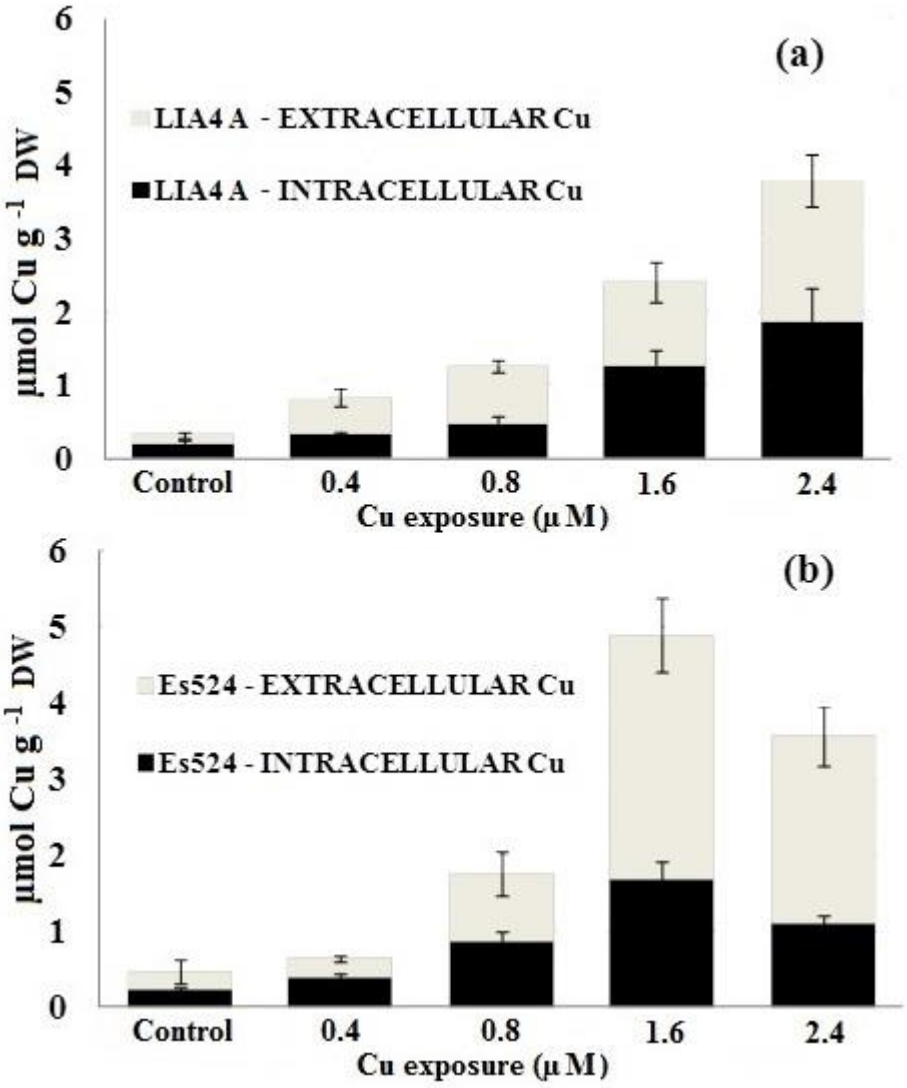

Figure 3.4. Extracellular and intracellular Cu concentrations in fresh weight biomass (FW) of the strains LIA4A (a) and Es524 (b). Error bars are ± 1 SD, n = 3.

The intracellular fraction, the highest accumulation was observed at 1.6and 2.4 µM Cu for Es524 and LIA4A ($p < 0.05$), respectively. For extracellular bound fraction, the highest levels were observed at 1.6 and 2.4 µM Cu for Es524 and LIA4A ($p < 0.05$), respectively (See Figure 3.4).

Comparison between strains evidenced similar intracellular Cu levels between Es524 and LIA4A, excepting under 2.4 µM Cu, where intracellular Cu was significantly higher in LIA4A than in Es524 ($p < 0.05$). Extracellular Cu levels were similar in the two strains at 0.4 and 0.8 µM Cu, but became significantly higher in Es524 compared to LIA4A strain at 1.6 and 2.4 µM Cu ($p < 0.05$). Just using the mean values, the percentage of intracellular Cu within total accumulation is presented in the Table 3.2; it can be observed that at the highest levels of exposure of 1.6 and 2.4 µM Cu, the intracellular fraction of the total Cu accumulated in Es524 decreases, and is also smaller that in LIA4A in all Cu treatments.

Table 3.2. Represent the percentage of intracellular concentrations (mean) of Cu within total accumulation observed in the *E. siliculosus* strains Es524 and LIA4A after Cu exposure for 10 days. The first row shows Cu treatments in concentrations expressed in terms of µM Cu.

	Control	0.4	0.8	1.6	2.4
Es524	61%	54%	49%	32%	29%
LIA4A	81%	39%	49%	53%	39%

3.3.3. Expression of enzymes in the pathway of glutathione-phytochelatin synthesis

A similar trend was observed for *GCL1* and *GCL2* genes encoding for glutamate cysteine ligase, in both strains (Figure 3.5; a,b). For Es524, there was up-regulation of *GCL1* and *GCL2* with increasing Cu exposure, with the highest levels of transcripts at 2.4 µM Cu. In LIA4A, the highest level of transcripts was at 0.8 µM Cu, followed by down-regulation at higher concentrations of Cu exposure.

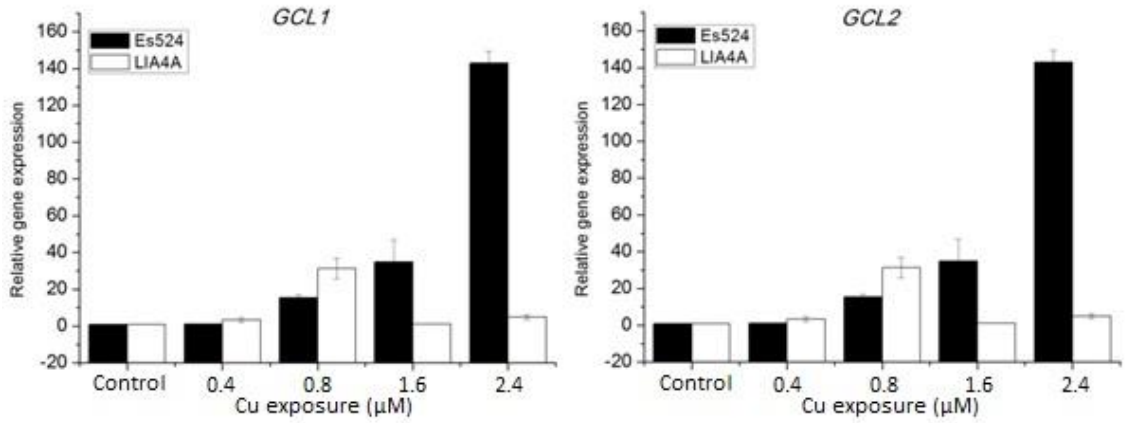

Figure 3.5. Pattern of gene expression in the two strains tested, LIA4A and Es524. Genes analysed are: glutamate cysteine ligase 1 and 2 (*GCL1*, 6a; *GCL2*, 6b. The expression was normalized to the expression of alpha tubulin (*TUA*), the housekeeping gene. Error bars are ± 1 SD, n = 3

The highest levels of *GS* transcripts were observed in LIA4A under control conditions and at 0.4 µM Cu; at higher concentrations there was down-regulation of *GS* in LIA4A (Figure 3.6). In the case of Es524, there was a trend of up-regulation of *GS* transcripts with increasing Cu exposure to 1.6 µMCu, with no change at 2.4 µM Cu.

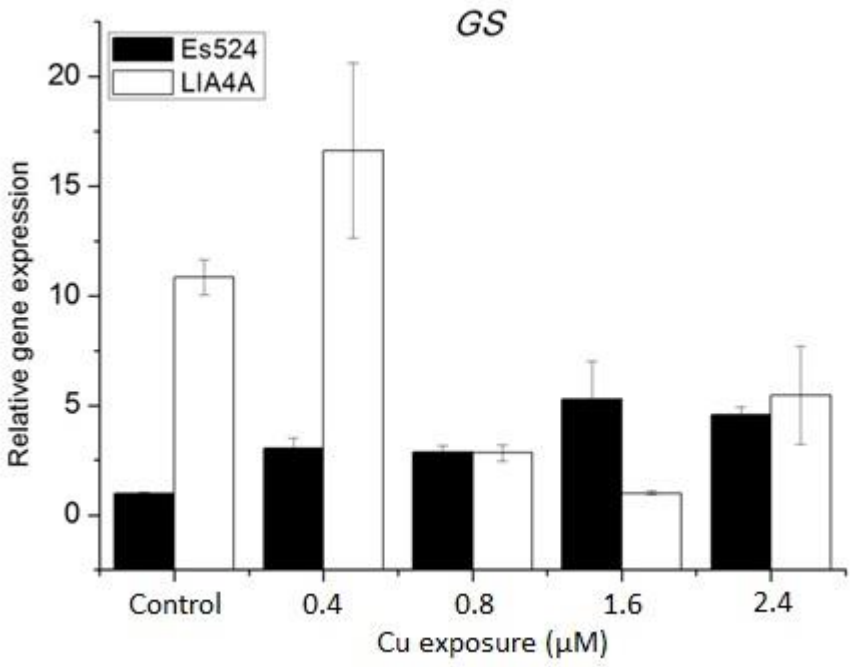

Figure 3.6. Pattern of expression of glutathione synthase (*GS*) in the two strains tested, LIA4A and Es524. The expression was normalized to the expression of alpha tubulin (*TUA*), the housekeeping gene. Error bars are ± 1 SD, n = 3.

For the gene encoding PCS there was up-regulation with increasing Cu exposure in Es524, with the highest levels of transcripts found at 2.4µM Cu (Figure 3.7). In comparison, for LIA4A, there was up-regulation of *PCS* at 0.4 and 0.8 µM Cu, followed by down-regulation at higher concentrations of Cu exposure, to levels measured in the control conditions.

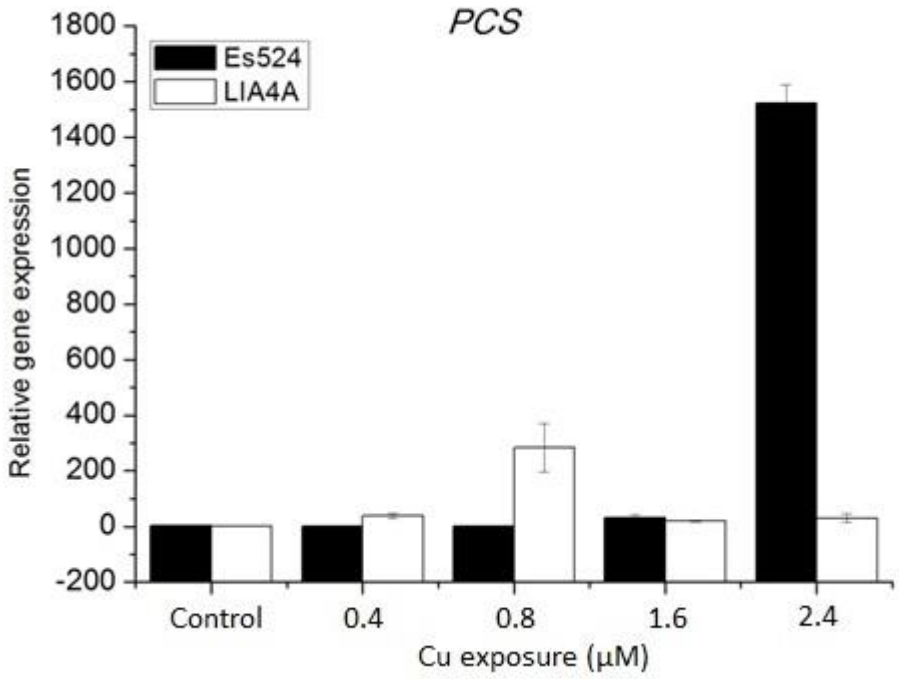

Figure 3.7. Pattern of expression of phytochelatin synthase (*PCS*) in the two strains tested, LIA4A and Es524. The expression was normalized to the expression of alpha tubulin (*TUA*), the housekeeping gene. Error bars are ± 1 SD, n = 3.

3.3.4. Glutathione and phytochelatin production under Cu exposure

Total glutathione concentrations significantly increased with Cu exposure in both strains (two-way ANOVA; $factor_{strain}$, $p < 0.05$; $factor_{Cu\ exposure}$, $p < 0.05$; interaction, $p < 0.05$). Except at 0.4 µM Cu, the concentations of total glutathione in LIA4 were either higher or similar to those in Es524 (Figure 3.8a). Concentrations of PC2 in Es524 were significantly different between Cu treatments (one-way ANOVA, $p < 0.05$). By the end of the experiment, the total concentration of PCs was significantly ($p < 0.05$) higher in Es524 than LIA4A under all treatments, including the control (Fig. 3.8a). Es524 exhibited significant increases in PC2 content at 0.4 µM Cu compared with the control ($p < 0.05$). At

higher Cu concentrations PC2 content remained at levels measured at 0.4 and 0.8 µM Cu. Although a pattern of increasing PC2 levels in LIA4A (Figure 3.8b), there were no significant differences between treatments (one-way ANOVA, $p > 0.05$). Longer chain PC3 was only found in Es524 (one-way ANOVA, $p < 0.05$), with highest content in controls ($p < 0.05$).

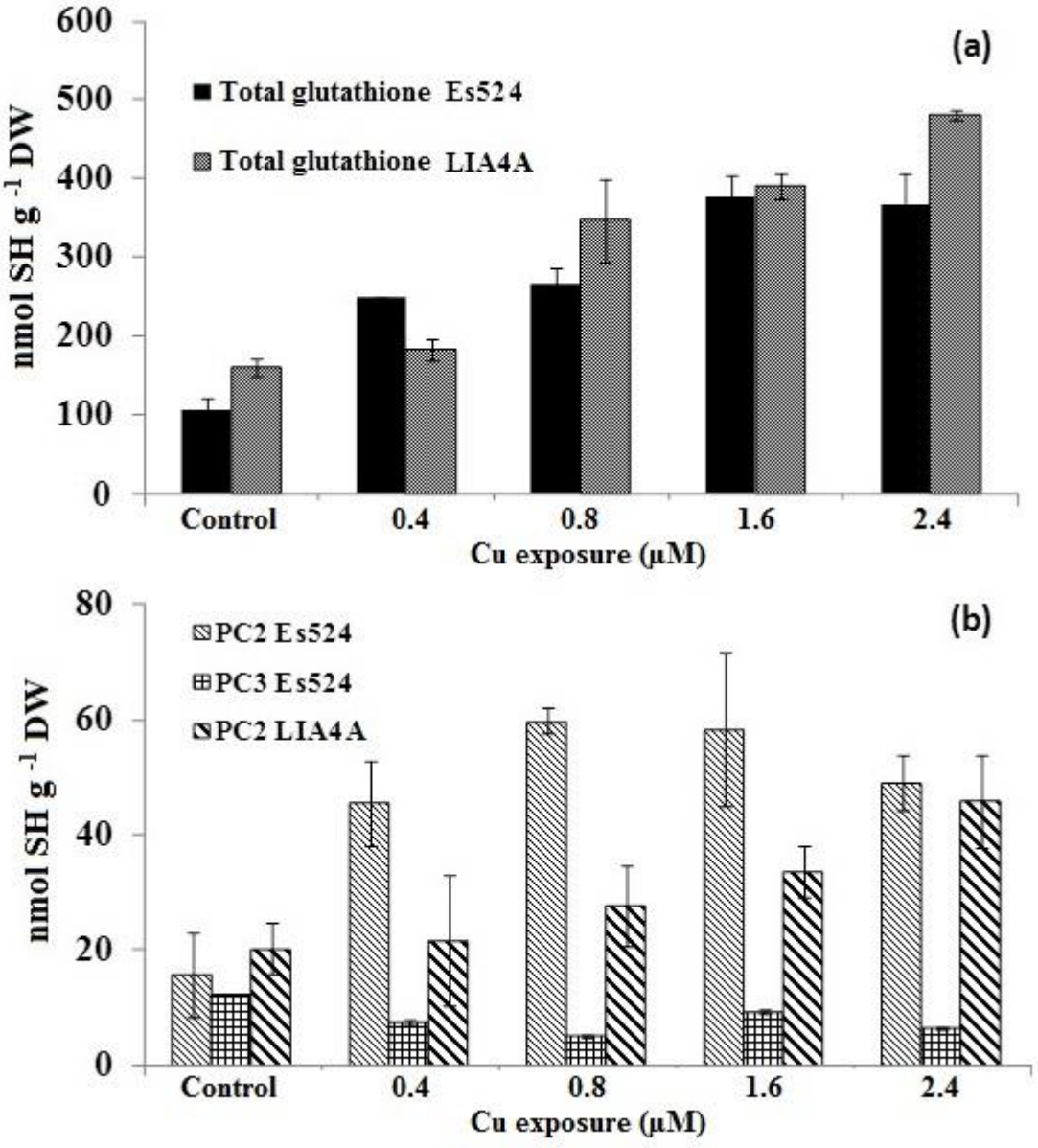

Figure 3.8. Concentrations of total glutathione (a) and phytochelatins (b) in the two strains of *Ectocarpus siliculosus*. Error bars are ± 1 SD, n = 3.

3.4. Discussion

The results presented here provide evidence for differential Cu-resistance in *Ectocarpus siliculosus* that is mediated by the extent of Cu contamination in the environment from where populations originate. Growth inhibition of Es524, from a Cu contaminated site, occurred at significantly higher Cu concentrations and to a lesser degree than that of LIA4A, isolated from site with no history of Cu contamination. These results of intra-specific variation are in general agreement with previously published studies on *E. siliculosus* and are likely to be inherited due to their history of Cu exposure. Hall (1980) observed that at 1.6 µM Cu for 14 days, a population of *E. siliculosus* sampled from a boat's hull coated with Cu-based antifouling paint had higher growth rates than a population isolated from a pristine site. More recently, Ritter et al. (2010) exposed two strains of *E. siliculosus* to chronic levels of Cu, Es32 from a location with no history of Cu pollution in Peru and strain Es524. After 10 d exposure, there was 70% cell death in Es32 at 0.8 µM Cu, whereas in Es524, they only observed significant cell death at 4µM Cu. The latter study did not evidence significant cell death at 2.4 µM Cu in Es524; however, our data suggests there is impairment in metabolism of Es524 as manifested in a decrease in RGRs at these levels of Cu exposure.

We observed that total Cu accumulation between strains was similar, with a common pattern of increase in parallel with greater Cu exposure. However, we found that Cu chelation in the cell wall was greater in Es524 than in LIA4A at high levels of Cu exposure (1.6 and 2.4 µM Cu). The effectiveness of metal adsorption in brown algae is related to the content and conformation of alginic acid in the cell wall plus other sulphated polysaccharides (Davis et al., 2003). The carboxylic groups between segments of polymers of guluronic (G) and mannuronic (M) acids forming alginate are the key targets for metal ions. Changes in M/G ratios can mediate metal binging capacity in brown algae (Haug,

1967), and have been observed to change under fluctuating environmental conditions (Venegas et al., 1993; Davis et al., 2003). Adsorption could also have been improved by the presence of epibionts, as observed in an increase in the amount and complexity of epiphytic bacterial communities in a Cu-polluted site in *Ulva compressa* in relation to a pristine location (Riquelme et al., 1997), which are believed to enhance chelation through exudation of organic compounds or cell-wall-binding (Hengst et al., 2010). We found that intracellular Cu (from total accumulation) was 53% and 39% in LIA4A, in comparison to 32% and 29% in Es524, at 1.6 and 2.4 µM Cu, respectively. The data suggests that Cu resistance in Es524 is importantly based stronger Cu-exclusion mechanisms than in LIA4A, which helps preventing Cu excess intracellularly and subsequent biological stress, as manifested in higher RGRs at 2.4 µM Cu exposure in Es524 compared to LIA4A. The ability of Es524 to maintain higher RGRs at external, and internal, concentrations of Cu, that inhibit more the growth of LIA4A, imply that this strain can more effectively detoxify Cu and maintain cellular Cu-homeostasis. Our results differ from Hall et al. (1979), which proposed that Cu defences in Cu-resistant strains of *E. siliculosus* were little- or non-related to exclusion mechanisms derived from metal adsorption, and more associated to intracellular defences and permeability of the cellular membrane. Instead, our data suggest that Cu-resistance can be importantly mediated by both: efficient mechanisms of exclusion, including adsorption, and intracellular defences.

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). The expression of the gene encoding *PCS* increased strongly in Es524 with Cu exposure; for example at 2.4 µM Cu there was a 1500-fold increase. In contrast, up-regulation of *PCS* in LIA4A showed a cut-off threshold at 0.8 µM Cu and then suffered down-regulation at higher levels of Cu

exposure. A similar pattern of expression was observed for glutathione related genes, *GCL1* and *GCL2*, and *GS*. Related information is lacking for other brown algae but comparisons can be made with other photoautrophs. For example, Zhao et al. (2010) agro-infected individuals of the non-tolerant plant *Agrostis palustris* with a *GCL* gene, and plants were watered with 15 mM $CdCl_2$ for 5 d; there was strong up-regulation of *GCL* and *PCS* in transgenic individuals compared to 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, and suggested PCs as an important chelating agent that helped decrease Cd toxicity intracellularly. Mellado et al. (2012) also observed a strong induction of GCL and GS in the green alga *Ulva compressa,* as manifested in the increase in the activity of both enzymes under 10 µM Cu. Even though intra-specific differences in the induction of GCL, GS and PCS is quite poor in the literature, we observed their patterns of expression were highly strain-specific, and highlight Es524 as better prepared in order to produce GSH and PCs under Cu stress.

The results presented here provide evidence for a differential pattern of expression in *GCL1 and GCL2* and *GS* genes between strains, and indicate that Es524 should produce more GSH. However, the concentrations of total glutathione (GSH+GSSG) increased under greater 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). Analyses of GSH and GSSG concentrations in LIA4A and Es524 under Cu exposure, reported in greater depth in Chapter IV, indicate that while the quantity of total glutathione might be similar in the two strains under Cu stress, the percentage of GSSG in the glutathione pool is significantly higher in LIA4A compared to Es524.

Backor et al. (2007) measured PCs levels in two (one wild-type and one metal-tolerant) strains of the green alga *Trebouxia erici* under Cu exposure of up to 10 µM for four weeks. These authors did not find higher PC levels in the tolerant strain and, moreover, at 5 µM Cu PCs content in this strain was significantly lower than in the wild-type, suggesting that PCs were not a major defence mechanisms mediating inter-population Cu resistance in this species. Our investigation provides the first evidence of induction and production of PCs induction in brown algae in response to Cu stress and, moreover, assessing intra-specific variation. We observed an increase in PC2 under Cu exposure only in the 'Cu tolerant' Es524, but not in LIA4A. Moreover, although levels did not increase with Cu exposure, longer chain dimers PC3 were only detected in Es524, but not in LIA4A. It is well established that the longer the PC, the more binding sites will be available in order to chelate metals (Cobbett, 2001). The information suggests that short PC2 might be 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, there is evidence that longer chain PCs can be induced in Es524 as manifested under Cd exposure, the strongest PCs inducer (Cobbett, 2000; Cobbett, 2001), which showed increased levels of dimers PC2 and PC3, and even PC4. Higher PCs content and longer chain length PCs have been previously detected in natural assemblages of *Fucus serratus* from a metal polluted environment (including Cu pollution), compared to biomass collected from pristine waters in south-west England (Pawlik-Skowronska et al., 2007). This information highlights PCs production as an important metal defence mechanism in brown algae, although with clear intra-specific plasticity. On the other hand, we observed PCs in controls of both strains (even PC3 in Es524); even though this might look surprising, there is evidence that PCs might play other roles differently than strictly metal chelators, such as participating in homeostatic regulation and storage of essential metals (Steffens et al., 1986; Tennstedt et al., 2009; Rea,

2012), 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).

In a recent study, Brunetti *et al.* (2011) measured PCs concentrations and several physiological parameters in a mutant of *Arabidopsis thaliana* that over-expresses *PCS* under Cd exposure, and compared its responses to that of wild-type individuals. The authors concluded that the greater production of PCs and higher degree of polymerization under high Cd exposure, compared to wild-type plants, prevented physiological dysfunction of the mutant. Similar conclusions can be drawn here; we observed higher expression of *GLC*, *GS* and *PCS*, greater PCs content and polymerization, and lesser effects in RGRs in the strain Es524 at the greatest levels of Cu exposure. Es524 originates from a location with several decades of Cu contamination resulting from the transport of wastewater from mining activities into a the coastal waters at Chanaral, Chile, and which has led to a severe reduction in species richness and modifications to the structure of intertidal communities in the vicinity (Correa et al., 1999; Medina et al., 2005). Moreover, Cu mining in this area is a result from high Cu levels in the Atacama Desert. Different records suggest that in addition to mining activities, Cu concentrations in coastal waters have been historically higher, even when anthropogenic impact was absent (Alpers and Brimhal, 1988; Maksaev et al., 2007). Our results support the idea that after a long history of exposure, Es524 could have undergone different genomic and/or epigenetic adaptations, making this strain more resistant to Cu. Now with the genome available for *E. siliculosus*, future seems promising in order to better understand the genomic and transcriptomic basis of metal stress metabolism in brown algae.

A long history of Cu exposure mediated the development of a tolerant ecotype (Es524) which, compared to a population originating from a pristine site (LIA4A), displays several attributes that allow growth and survival in a highly Cu-impacted

environment: better exclusion of Cu by efficient extracellular adsorption, stronger up-regulation of genes encoding enzymes intimately involved in the pathways of GSH-PCs synthesis, greater production of PCs with greater levels of polymerization (PC2 and PC3), all of which permits the maintenance of higher growth rates at the highest concentrations of bioavailable Cu used in this investigation. This is the first study to analyse concurrently, metal exclusion, intracellular metal content, glutathione and PCs concentrations, and the transcriptional expression of genes encoding the enzymes catalysing GSH-PC synthesis in brown algae. These findings help to improve our understanding of the mechanisms involved in environmentally-induced inter-population adaptations to Cu stress in an economically and commercially important group of marine chloroxygenic organisms.

Chapter IV

Inter-population responses to copper stress relates to divergent inherited antioxidant strategies in the model brown alga *Ectocarpus siliculosus*

4.1. Introduction

Reactive oxygen species (ROS) are important products of plant and algal metabolism. ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($\bullet O_2^-$), organic hydroperoxide (ROOH), alkoxy radicals (RO•), peroxy radicals (ROO•), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$), are signalling molecules and take part in important processes such as modulating gene expression and controlling the activity of protein complexes for defence purposes (Apel and Hirt, 2004). However, biological and abiotic stressors can induce over-production of ROS causing oxidative stress (Halliwell and Gutteridge, 2007), leading to oxidative damage including DNA mutations, lipid peroxidation, covalent modification of proteins and oxidation of photosynthetic pigments (Zuppini et al., 2010). Excessive concentrations of metals such as Cd, Cu and Pb are important inducers of ROS over-production; for instance, although Cu is an essential metal, high concentrations can affect the redox potential of redox couples associated with NADPH-dependent oxidase enzymes, producing electron transfer to oxygen and subsequent over-production of $\bullet O_2^-$, which is precursor of H_2O_2 and a less reactive ROS (Gonzalez et al., 2010). Moreover, free or bound Cu(II) can be reduced to Cu(I) by $\bullet O_2^-$ and react with available H_2O_2 in the Fenton reaction to produce the highly oxidizing •OH (Navari-Izzo et al., 1999). To counteract the effects of high ROS production, cells have a battery of antioxidant molecules such as glutathione (GSH), ascorbate (Asc), phenolic compounds and carotenoids, and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione peroxidase (GPx), and catalase (CAT) (Apel and Hirt, 2004).

The enzyme SOD is the first line of defence against ROS, catalysing the dismutation $\bullet O_2^-$ into O_2 and H_2O_2 (Ken et al., 2005). Therefore, the action of different SOD isozymes (MnSOD in mitochondria, FeSOD in chloroplasts, and CuZnSOD in the

cytosol) provides important protection for cells (Fridovich, 1997). In addition to decomposition of $\bullet O_2^-$, high SOD activity can lead to an increase in the levels of H_2O_2. To counteract H_2O_2, cells use peroxidases, of which CAT is considered to be one of the most important, to catalyse the decomposition of H_2O_2 into H_2O and O_2 (Chelikani et al., 2004).

The antioxidant characteristics of glutathione are related to the continuous thiol-sulfide interactions. GSH is oxidized by ROS to produce the disulfide form of glutathione (GSSG), which is later recycled to GSH by the NADPH-dependent enzyme GR (Noctor et al., 2012). Asc transfers a single electron to ROS to produce monodehydroascorbate (MDHA), which later dismutates to dehydroascorbate (DHA), the most stable form of oxidized ascorbate (Biaglow et al., 1997). DHA can also react with GSH in the presence of the enzyme dehydroascorbate reductase (DHAR) to recycle Asc, the basis of the glutathione-ascorbate cycle (Noctor et al., 2012). Asc can also be oxidized to MDHA by H_2O_2 in an APx-catalysed reaction (Asada, 1992). Additionally, phenolic compounds are active antioxidants due to the electron donation capacity of the acidic hydroxyl group. Oxygen radicals oxidize phenolic compounds to phenoxyl radicals, which are generally less reactive than ROS (Pedrielli et al., 2001).

Brown seaweeds are the main primary producers at the base of trophic networks in coastal waters and estuaries , providing food and shelter for a high diversity of marine organisms (Sáez et al., 2012b). They are regularly exposed to both natural and anthropogenic environmental stressors including high irradiance, temperature and salinity fluctuations, and chemical pollutants such as metals, all of which can produce oxidative stress (Collen and Davison, 1999; Pinto et al., 2003). Certain brown seaweeds species display high levels of resistance to metal stress, and in addition to antioxidant defences they also have several other protective mechanisms associated with strong reducing agents

and metal chelators (Pawlik-Skowronska et al., 2007; Sáez et al., 2012a). To date, much of the research has focussed on inter-specific differences in metal resistance although there is some limited evidence for intra-specific variation in the responses of brown seaweeds to metal exposure. For example, there is evidence for the development of metal resistant 'ecotypes' in at least two species of brown seaweeds, *Fucus serratus* and *Ectocarpus siliculosus* (Nielsen et al., 2003; Ritter et al., 2010). The divergent phylogenetic evolution of brown algae that separates them from other photosynthetic organisms such as red and green algae, and vascular plants (Biaglow et al., 1997), highlights the importance of investigating the mechanisms of metal resistance in this important group of marine photoautotrophs.

The filamentous alga *E. siliculosus* has recently been proposed as a model organism for brown algae (Peters et al., 2004a). The rationale for this choice is the almost two hundred years of research on different aspects of its biology plus several features that make it suitable for experimentation, for example, its small size and fast growth, ease of culturing and the ability to complete the entire life cycle in the laboratory in a relatively short period time (Charrier et al., 2008). Strains of *E. siliculosus* have been isolated from different locations around the world and maintained in culture (Dittami, Simon M. et al., 2011), providing the opportunity to investigate inter-population responses to metal stress. The focus of this chapter is to assess the antioxidant responses of three strains of *E. siliculosus* under different levels of Cu exposure, two strains originating from Cu-polluted locations and one from a location with no history of metal contamination. To this end, indicators of oxidative stress H_2O_2 and lipid peroxidation, and concentrations of the pigments chlorophyll *a* and *c*, and fucoxanthin, were measured. Reduced and oxidized forms of the antioxidants glutathione and ascorbate were quantified to investigate the glutathione-ascorbate cycle and phenolic compounds were measured to evaluate their

importance as antioxidants in this species. Finally, the activities of antioxidant enzymes SOD, APx and CAT were also determined.

4.2. Materials and Methods

4.2.1. Strain selection and culture conditions

Three strains of *E. siliculosus* isolated from populations with different histories of Cu exposure were used: Es524, originating from Palito la Boca, Chañaral, Chile (Cu polluted site); REP10-11, from Restronguet Creek, Cornwall, England (metal, including Cu polluted site); and LIA4A, from Lon Liath, Scotland (pristine site). Strains were grown separately in 2 L polycarbonate bottles containing seawater enriched with Provasoli solution (Provasoli and Carlucci, 1974). Culture conditions were 15 °C, 45 µmol photons m^2 s^{-1}, on a 14:10h light/dark cycle, and aeration to avoid CO_2 depletion and maintain material in suspension. Once sufficient biomass had been obtained, material was transferred to the chemically defined synthetic seawater medium Aquil (Morel et al., 1979), but without the addition of a metal chelating agent, and acclimatized for 10 d before experimentation (Gledhill et al., 1999).

4.2.2. Cu exposure experiments

One gram of fresh biomass of each strain was transferred to polycarbonate flasks containing 125 mL of Aquil enriched with $CuSO_4.5H_2O$, with 3 replicates per treatment. Nominal total Cu concentrations [Cu_T] were 0, 0.4, 0.8, 1.6 and 2.4 µM, which 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] software based on the

Debye-Huckel equation, using a temperature of 288 K, pH 8.2 and atmospheric pCO_2 of 3.5×10^{-4} atm (Nielsen et al., 2003). The exposure period was 10 d and the medium was replaced every 2 d to compensate for any release of metal-complexing ligands by algal cells (Gledhill et al., 1999; Nielsen et al., 2003). After the period of exposure, the biomass was collected, frozen in liquid nitrogen and stored at -80 °C for further analyses.

4.2.3. Effects of Cu-mediated oxidative stress

Concentrations of H_2O_2 were measured according to Sergiev et al. (1997), with the method modified for a plate reader (VERSA MAX). Frozen fresh biomass (100 mg) was homogenized in 1 mL of 10% trichloroacetic acid (TCA) in a 1.5 mL centrifuge tube. Glass beads (c. 5, 3 mm diameter) were added and the tubes were vortexed for 5 min. The homogenate was centrifuged at 21,000 g (Sanyo Hawk 15/05) for 10 min and 50 µL of supernatant were added to each well. 150 µL of 50 mM potassium phosphate buffer (pH 7.0) and 100 µL of 1 M potassium iodide were added to each well, and the absorbance was recorded at 390 nm. Concentrations between 0 and 3200 ng mL^{-1} of H_2O_2 in 10% TCA solution were used as standards. Lipid peroxidation (LPX) was measured according to Heath and Packer (1968) with modifications for a plate reader. Algal biomass was extracted as described for H_2O_2 measurements: 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. Malondialdehyde (MDA) was used as a standard with concentrations between 0 and 70 µM per well.

Chlorophyll *a* (Chl*a*) and *c* (Chl*c*), and fucoxanthin (Fx) were extracted according to Seely et al. (1972). Fresh biomass of *E. siliculosus* (200 mg) was placed in a

1.5 mL glass test-tube to which 800 µL of dimethyl sulfoxide (DMSO) was 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 of DMSO:water and pigment concentrations then determined spectrophotometrically (Jenway 7315) at different wavelengths. Pigment concentrations are expressed in moles per gram of fresh biomass and calculated using the following equations:

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

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

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

4.2.4. Concentrations of antioxidants

Reduced (GSH) and oxidized (GSSG) glutathione were measured using a modification of the method of Queval and Noctor (2007). 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. The supernatant was removed and neutralized with 5 M K_2CO_3 to a 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, 10 µL of 0.34 mM NADPH, 0.4 mM DTNB, and 1 unit of GR (Sigma Aldrich, G3664). The rate of absorbance change was measured at 412 nm for 5 min; GSH was used as a standard in 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. Extracts were then centrifuged at 21,000 g for 5 min at 4 °C; GSSG was measured as for total GSH using GSSG as a standard in concentrations between 0 and 0.5 µM. GSH was calculated by subtracting GSSG from total GSH. Ratios of GSH:GSSG were used as a measure of oxidative stress, according to Noctor et al. (2012).

Concentrations of ascorbate (Asc) and dehydroascorbate (DHA) were measured using ferric tripyridyl triazine (FRAP reagent) as described by Benzie & Strain (1999), with the assumption that nothing else in the samples other than ascorbate reacts with it. Biomass was extracted as for glutathione but without neutralizing the supernatant. To measure Asc, 10 µL of extracts were placed in each well of a 96-well plate, 290 µL of FRAP reagent then added to each, and the absorbance measured immediately at 593 nm ; with ascorbate standards the colour develops immediately and does not change significantly within the first minute. To measure 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 Asc values from total ascorbate. L-ascorbate was used as a standard.

To measure total phenolic compounds, the protocol was modified from van Alstyne (1995). Fresh biomass (100 mg) was added to a 15 mL tube 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. Phloroglucinol (0 to 0.17%) was used as standard.

4.2.5. Activities of antioxidant enzymes

A single extraction, modified from Collen and Davison (1999), was used to produce an extract for the measurement of the activity of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APx) and catalase (CAT). Frozen biomass (0.2 g) was extracted with 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 (c. 5, 3 mm diameter) were added and the tubes then vortexed for 10 min at 4 °C. Extracts were centrifuged at 21,000 g for 5 min at 4 °C, and the supernatants stored overnight at -20 °C or for 1 h at -80 °C to encourage aggregation of biological membranes. Samples were centrifuged again at 21,000 g for 5 min at 4 °C. Extracts were stored at -80 °C to await analyses of enzyme activity.

The activity of SOD was measured according to McCord and Fridovich (1969), with modifications for a plate reader (50 µL) . The assay mixture contained 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}. A 50 µL extract 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 water diluted commercial SOD (Sigma Aldrich, G5389) 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).

The activities of APx and CAT were determined according to 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_2O_2 was added to a final concentration of 0.1 mM. APx activity was measured at 20 °C using the decrease of absorbance at 290 nm monitored over 30 s. For CAT 50 µL of extract was added to 700 µL 50 mM potassium phosphate buffer, pH 7.0. The reaction was started by the addition of 11 mM H_2O_2 and the activity calculated from the decrease in absorbance at 240 nm over 1 min at 20 °C.

4.2.6. Statistical analyses

The statistical package used was SPSS v.5. All data were checked for normality and homogeneity of variances using the Shapiro-Wilk and Bartlett tests, respectively. Two-way ANOVA followed by post-hoc Tukey test (at 95% confidence) were performed to identify significant differences in most of the biochemical parameters between the main factors: *E. siliculosus* strains (3 levels) and Cu treatments (5 levels) and their interactions.

For chlorophyll and ascorbate, a multivariate ANOVA (MANOVA) was applied. The factors were: the *E. siliculosus* strains (3 levels) and Cu treatment (5 levels). The dependent variables were: Asc and DHA for ascorbate; and Chla and Chlc for chlorophyll.

4.3. Results

4.3.1. Effects of Cu-mediated oxidative stress

Concentrations of H_2O_2 increased with Cu exposure and differed between Es524 (Cu polluted site in Chile), LIA4A (pristine site in Scotland) and REP10-11 (Cu polluted site in England) (two-way ANOVA, factor Cu exposure, $p < 0.05$; factor strain, $p < 0.05$; interaction, $p < 0.05$). Levels of H_2O_2 were always significantly higher in LIA4A compared to Es524 and REP10-11 ($p < 0.05$) when exposed to Cu (Figure 4.1a). For Es524, there was a significant decrease in the levels of H_2O_2 when exposed to 0.4, 0.8, and 1.6 µM Cu, compared to the controls ($p < 0.05$); at 2.4 µM Cu, H_2O_2 in Es524 showed no significant difference to the control level ($p = 0.595$). There was no significant difference in the levels of H_2O_2 in REP10-11 up to and including 0.8 µM Cu ($p > 0.05$); at 2.4 µM Cu, levels of H_2O_2 were significantly higher than all other treatments ($p < 0.05$) except 1.6 µM Cu ($p < 0.05$), and. Levels of H_2O_2 in REP10-11 were significantly higher than in Es524 when exposed to 1.6 and 2.4 µM Cu ($p < 0.05$).

Significant differences were found in the extent of lipid peroxidation (LPX) between strains and among Cu exposure treatments (two-way ANOVA, factor Cu exposure, $p < 0.05$; factor strain, $p < 0.05$; interaction, $p < 0.05$). LPX was always higher in REP10-11 than in Es524 and LIA4A ($p < 0.05$), except at 1.6 µM Cu ($p = 0.78$) (Figure 4.1b). There were no significant differences between LPX levels in Es524 and LIA4A ($p > 0.05$), except at 2.4 µM Cu, where LPX levels were higher in LIA4A ($p < 0.05$). In Es524, LPX concentrations did not differ significantly between treatments ($p > 0.05$).

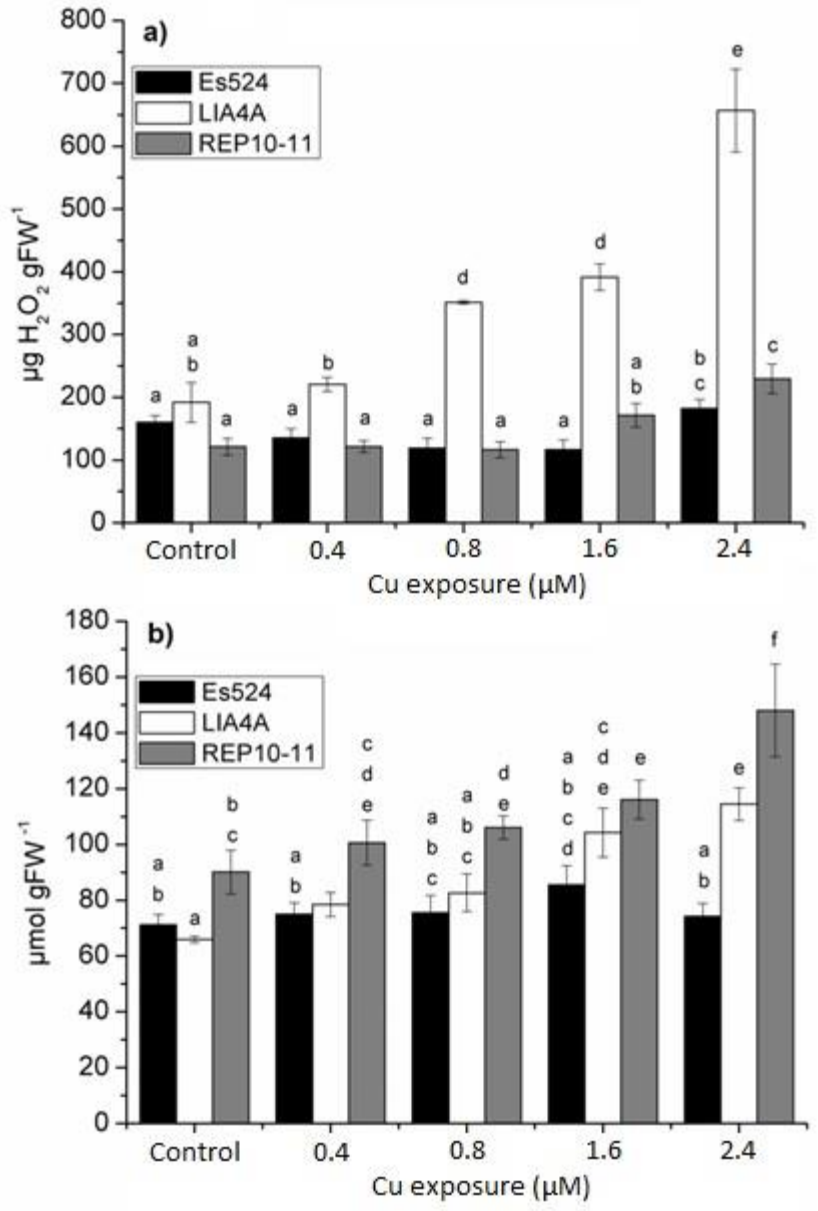

Figure 4.1. Oxidative stress parameters measured in three strains of *Ectocarpus siliculosus*; REP10-11 collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. Strains exposed to different concentrations of Cu for 10 days. a) Hydrogen peroxide. b) Lipid peroxidation. Different letters above bars represent significant differences at the 95% confidence level ($p < 0.05$). Error bars are ± 1 SD, $n = 3$.

Multivariate tests revealed significant differences for chlorophyll *a* (Chl*a*) and *c* (Chl*c*) between strains, Cu exposure and their interaction (MANOVA, Pillai's Trace, $p < 0.0001$). Levels of Chl*a* were always higher than Chl*c* for all strains ($p < 0.05$); however, the content of Chl*a* and Chl*c*, and the pattern among Cu treatments, differed between strains. For Chl*a*, although significant differences were found for several interactions, only in the cases of LIA4A and REP10-11 did they present patterns that could be attributed to Cu exposure (Figure 4.2a). At 2.4 µM Cu, Chl*a* levels in LIA4A and REP10-11 were significantly lower than for all other treatments ($p < 0.05$). In Es524, Chl*a* did not change significantly with Cu exposure ($p > 0.05$), except at 0.4 µM Cu, where concentrations were significantly lower than at all other treatments ($p < 0.05$). Levels of Chl*c* in LIA4A decreased significantly with increasing Cu exposure, with the lowest levels at 2.4 µM Cu ($p < 0.05$). No discernible patterns or significant changes in Chlc with Cu exposure were observed in REP10-11 and Es524, with the exception of the significantly lower concentrations at 1.6 µM Cu ($p < 0.05$) (Figure 4.2a). For fucoxanthin, there were significant differences between strains but not between treatments (two-way ANOVA, factor strains, $p < 0.05$; factor Cu exposure, $p = 0.143$), However, the interaction term was significant ($p < 0.05$) and can be accounted for by the variation between strains within Cu treatments and the significant decline in fucoxanthin at 1.6 and 2.4 µM Cu in LIA4A and significant increase at 2.4 µM Cu compared with controls (Figure 4.2b)

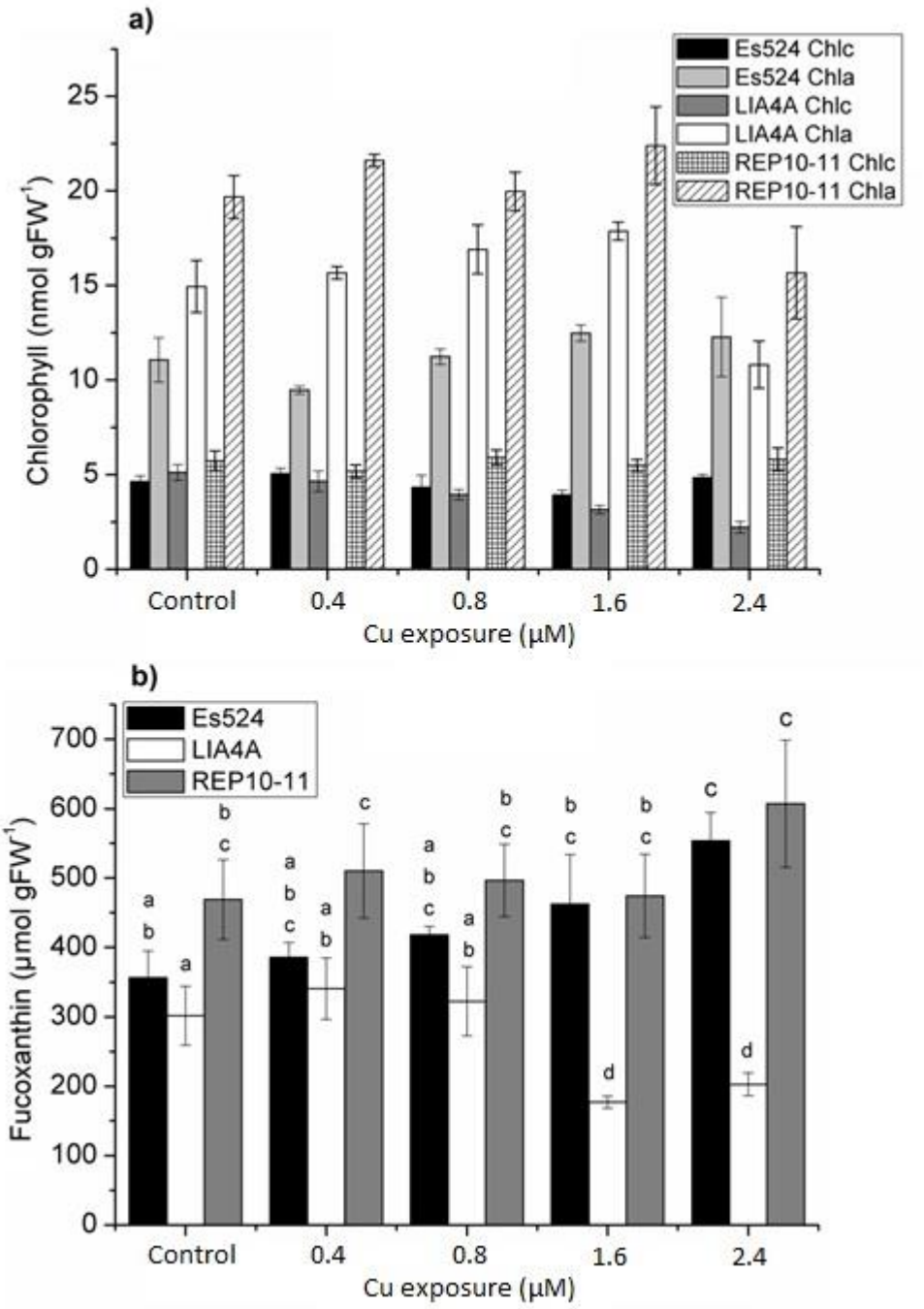

Figure 4.2. Pigments in three *Ectocarpus siliculosus* strains assessed after different levels of Cu exposure; REP10-11collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. a) content of chlorophylls *a* (Chl*a*) and *c* (Chl*c*). b) levels of fucoxanthin. Different letters above bars represent significant differences at the 95% confidence level ($p < 0.05$). Error bars are ± 1 SD, $n = 3$.

4.3.2. Levels of antioxidant compounds

The results of the ANOVA show a non-significant difference between strains, a significant difference between treatments and a significant interaction term (two-way ANOVA, factor strain, $p = 0.41$, factor Cu exposure, $p < 0.05$; interaction, $p < 0.05$). The highest levels of total glutathione in controls, and at 0.4 and 0.8 µM Cu, were found in LIA4A, in relation to Es524 and REP10-11 ($p < 0.05$) (Figure 4.3). In contrast, at 1.6 and 2.4 µM Cu, total glutathione was significantly lower in LIA4A than in Es524 and REP10-11 ($p < 0.05$). Between Es524 and REP10-1, at 0.4 and 0.8 µM Cu, total glutathione was significantly higher in REP10-11 ($p < 0.05$), while at 1.6 and 2.4 µM Cu, total glutathione levels were higher in Es524 ($p < 0.05$). Relationships between reduced (GSH) and oxidized (GSSG) glutathione could be observed in their ratio (GSH:GSSG) (Table 4.1). The GSH:GSSG ratios showed a clear pattern of decrease with increasing Cu exposure in LIA4A; at 1.6 and 2.4 µM Cu, greater GSSG than GSH in LIA4A resulted in ratios < 1, and significantly lower than in Es524 and REP10-11 ($p < 0.05$). In Es524, a decrease in GSH:GSSG ratios with greater Cu exposure was also observed, excepting at 100 µM Cu, where the ratio was significantly higher than for the rest of the treatments ($p < 0.05$); although the ratio in Es524 was lowest at 2.4 µM Cu, it never went below 1. The strain REP10-11 displayed the highest ratios at 0.4 and 0.8 µM Cu, significantly higher than for the rest of the treatments ($p < 0.05$); at 1.6 and 2.4 µM Cu, GSH:GSSG ratios decreased in REP10-11, and were not significantly different to those in the controls ($p > 0.05$).

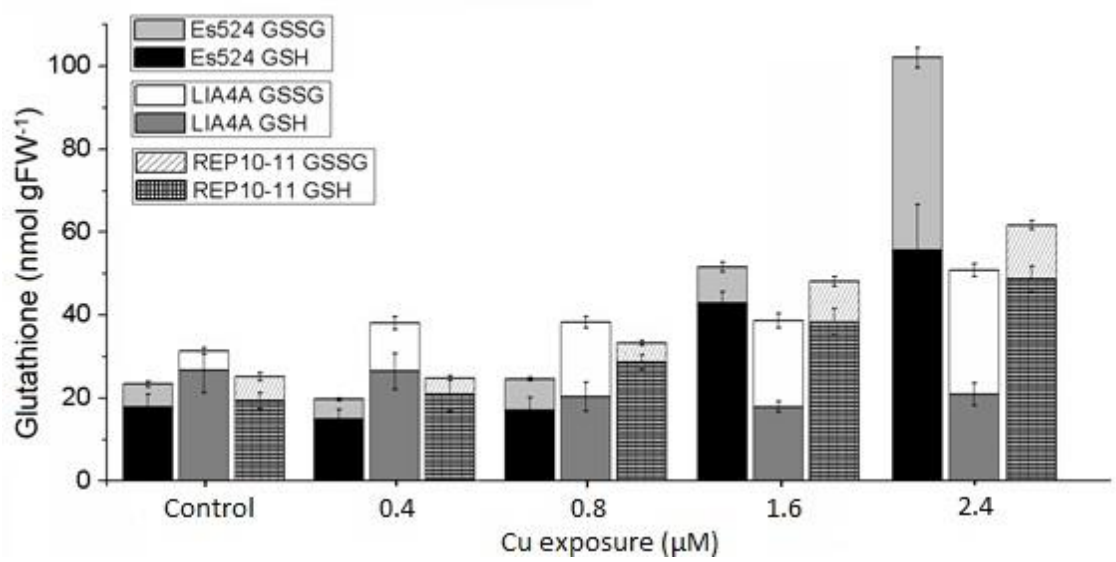

Figure 4.3. Levels of reduced (GSH) and oxidised (GSSG) glutathione in three *Ectocarpus siliculosus* strains assessed after different levels of Cu exposure; REP10-11 collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. Error bars are ± 1 SD of GSH and GSSG, separately, $n = 3$.

Table 4.1. Ratios of reduced and oxidised glutathione (GSH:GSSG) of the *Ectocarpus siliculosus* strains assessed under the different Cu treatments. Errors are ± SD, n = 3.

	Es524	**LIA4A**	**REP10-11**
Control	3.20 ± 0.69	5.9 ± 0.04	3.43 ± 0.50
0.4 µM Cu	3.15 ± 0.71	2.28 ± 0.08	5.37 ± 1.23
0.8 µM Cu	2.31 ± 0.51	1.13 ± 0.13	6.29 ± 0.73
1.6 µM Cu	4.99 ± 0.43	0.87 ± 0.12	3.97 ± 0.81
2.4 µM Cu	1.21 ± 0.30	0.70 ± 0.06	3.77 ± 0.10

Multivariate analyses demonstrated significant differences in the levels of ascorbate (Asc) and the oxidised form dehydroascorbate (DHA) between strains, Cu exposure and their interactions (MANOVA, Pillai's Trace, $p < 0.0001$). The strains Es524 and REP10-11 showed an increase in the levels of Asc with increasing Cu exposure, with Es524 having the highest Asc concentrations ($p < 0.05$). In LIA4A showed an increase in Asc levels increased up to 2.4 µM, but significantly decreased, thereafter ($p < 0.05$) (Figure 4.4). Asc levels in REP10-11 were only higher than in LIA4A at 1.6 and 2.4 µM Cu ($p < 0.05$). There were similar patterns in DHA content in the strains assessed; DHA levels in Es524 did not change significantly until 1.6 µM Cu, but at 2.4 µM Cu levels increased and were significantly higher than the rest of the treatments in Es524, and also in LIA4A and REP10-11 ($p < 0.05$) (Figure 4.4). The highest levels of DHA in REP10-11 and LIA4A were measured at 1.6 and 2.4 µM Cu, respectively ($p < 0.05$).

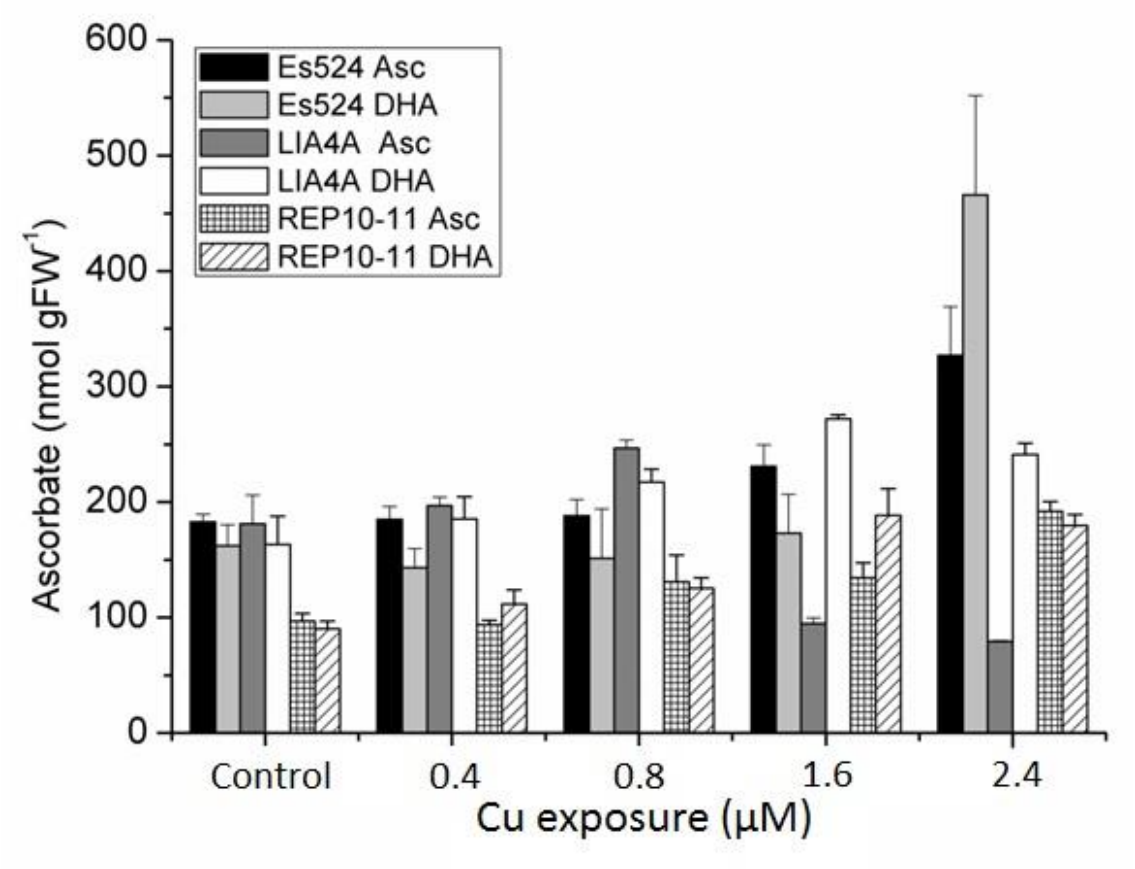

Figure 4.4. Levels of ascorbate (Asc) and dehydroascorbate (DHA) in three *Ectocarpus siliculosus* strains assessed after different levels of Cu exposure; REP10-11 collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. Error bars are ± 1 SD, $n = 3$.

There were no significant differences between levels of phenolic compounds between strains (two-way ANOVA, factor strain, $p = 0.102$; interaction, $p < 0.05$) and Cu treatments (factor Cu exposure, $p = 0.064$), probably because of the interaction term of decrease in LIA4A at 0.8 µM Cu; however, the latter is significant at the 90% confidence interval given by the pattern of increase with greater Cu exposure observed in all the strains measured (see Figure 4.5). At 2.4 µM Cu, all strains had significant higher levels of phenolic compounds than their controls ($p < 0.05$). Although there was a pattern of increase in phenolic compound levels in LIA4A between 0.8 and 1.6 µM Cu, the content was always significantly lower than in Es524 and REP10-11 at the same Cu exposure ($p < 0.05$).

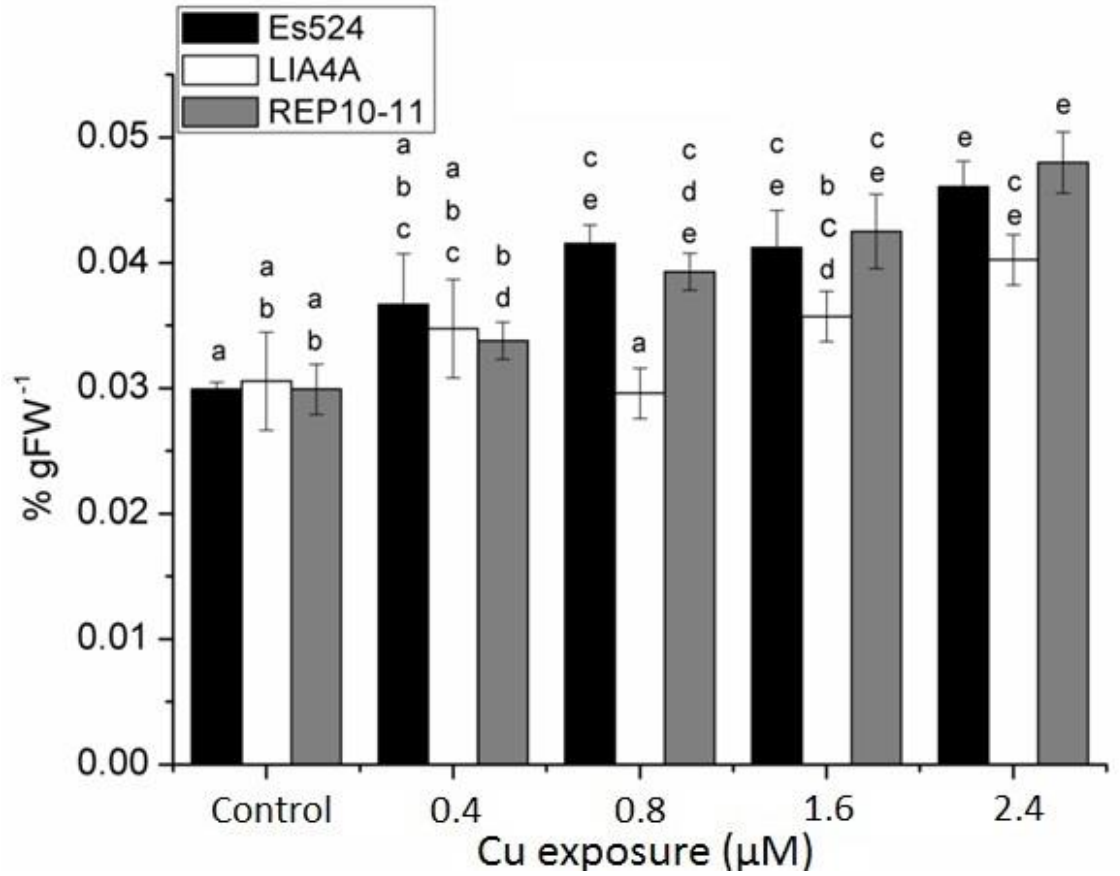

Figure 4.5. Levels of phenolic compounds, expressed in percentage (%) of the standard phloroglucinol, in three *Ectocarpus siliculosus* strains assessed after different levels of Cu exposure; REP10-11 collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. Different letters in bars represent significant differences at the 95% confidence level ($p < 0.05$). Error bars are ± 1 SD, $n = 3$.

4.3.3. Activities of antioxidant enzymes after Cu exposure

The activity of superoxide dismutase (SOD) was significantly different between strains and Cu exposure (two-way ANOVA, factor strain, $p < 0.05$; factor Cu exposure, $p < 0.05$; interaction, $p < 0.05$). In Es524, activity of SOD was always significantly higher than controls and greater than LIA4A and REP10-11 when exposed to Cu ($p < 0.05$), excepting at 2.4 µM ($p > 0.05$) (Figure 4.6a). In LIA4A, SOD activity increased with Cu exposure, with the highest levels at 2.4 µM Cu ($p < 0.05$). SOD activity in REP10-11 did not differ significantly between treatments ($p > 0.05$).

Although the activity of ascorbate peroxidase (APx) was significantly different for the strains assessed (two-way ANOVA, factor strain, $p < 0.05$; interaction, $p < 0.05$) there was no clear pattern between them (Figure 4.6b). However, there was a similar trend of increase in the activity of APx for all strains with increasing Cu exposure (factor Cu exposure, $p < 0.05$). For all strains, APx activity at each level of Cu exposure was significantly higher than in their controls ($p < 0.05$), with the highest activity at 2.4 µM Cu ($p < 0.05$).

There were significant differences in the activity of catalase (CAT) between strains and also at different levels of Cu exposure (two-way ANOVA, factor strain, $p < 0.05$; factor Cu exposure, $p < 0.05$; interaction, $p < 0.05$). In LIA4A there were no significant differences in CAT between treatments ($p > 0.05$) (Figure 6c). In REP10-11, there were also no significant differences in CAT between Cu exposure ($p > 0.05$), excepting at 2.4 µM Cu, where CAT was significantly higher than in the rest of the treatments. The activity of CAT in Es524 increased with greater Cu exposure; excepting at 0.8 µM Cu, the levels of CAT in Cu treatments were all significantly higher than the controls ($p < 0.05$), with the highest levels at 2.4 µM Cu ($p < 0.05$).

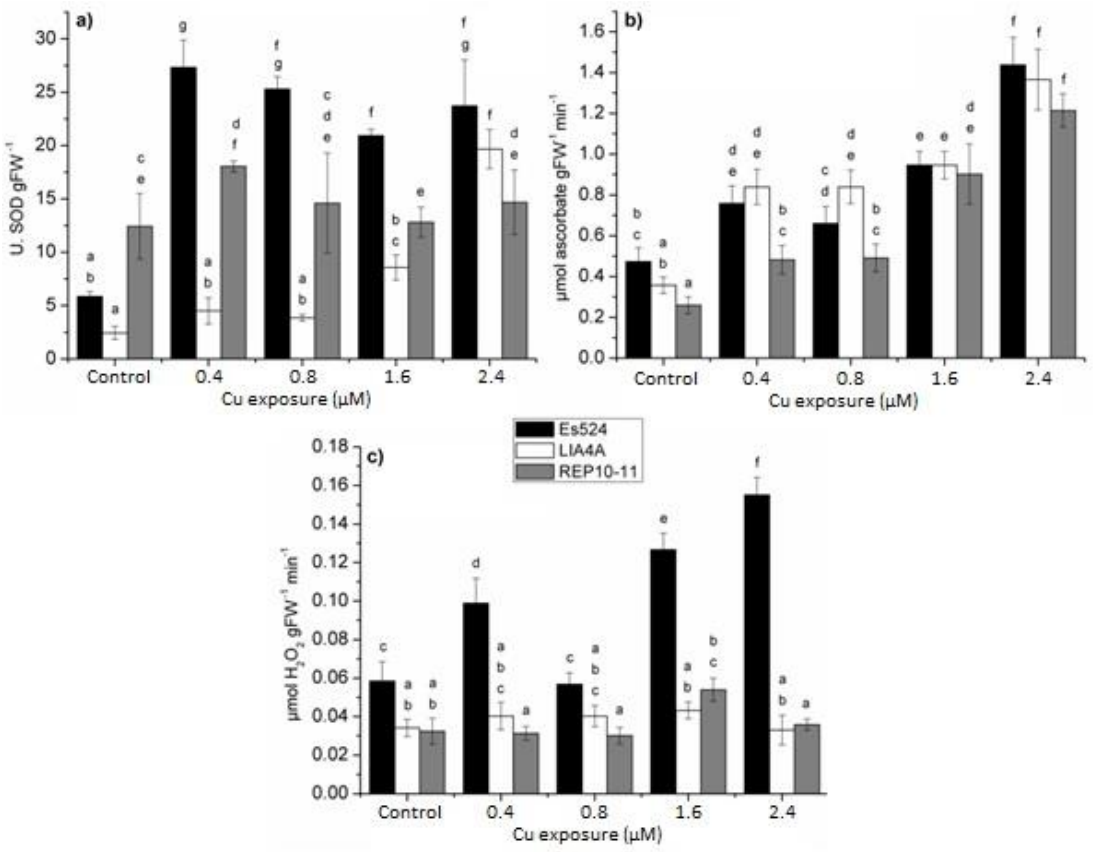

Figure 4.6. Activity of antioxidant enzymes in three *Ectocarpus siliculosus* strains assessed after different levels of Cu exposure; REP10-11 collected from a metal (including Cu) polluted location in England, Es524 from a Cu polluted site in Chile, and LIA4A from a pristine location in Scotland. a) superoxide dismutase. b) ascorbate peroxidase. c) catalase. Different letters in bars represent significant differences at the 95% confidence level ($p < 0.05$). Error bars are ± 1 SD, $n = 3$.

4.4. Discussion

Several studies have observed that following chronic or acute Cu exposure, the levels of ROS increase above homeostatic levels, a condition that can cause oxidative damage, which can be manifested in an increase in the levels of lipid peroxidation (e.g. Correa et al., 1999; Contreras et al., 2005; Contreras et al., 2009; Gonzalez et al., 2010). In our investigation, the strain LIA4A (from a pristine site in Lon Liath, Scotland) had the highest levels of the ROS H_2O_2 under increasing levels of Cu exposure, followed by REP10-11 (from a Cu-polluted site in Restronguet, England), while the strain Es524 (from a Cu-polluted site in Chañaral, Chile) did not seem to show Cu-induced elevation of H_2O_2 levels. The molecule H_2O_2 is one of the less harmful types of ROS; therefore, higher lipid peroxidation (LPX) levels in REP10-11 might be related to other oxygen radicals in excess. LPX has also been recorded by Contreras et al. (2009) in the brown algae *Lessonia nigrescens* and *Scytosiphon lomentaria* exposed to up to 1.6 µM Cu; these authors found a systematic increase in LPX in *L. nigrescens*, which in turn was lower in *S. lomentaria*, showing species-specific resistance to Cu-induced excess of ROS. In our study, the *E. siliculosus* strain LIA4A showed the highest damage in terms of LPX levels, whereas Es524 did not display abnormal LPX in response to the same Cu levels.

The principal photosynthetic pigment in brown algae, chlorophyll *a* (Chl*a*), presented different levels in the strains assessed; the strains REP10-11 and Es524 had the highest and lowest levels, respectively. *E. siliculosus* can cover a widespread region of the coastal subtidal and intertidal and, moreover, grow attached to a hard substrate, as an epiphyte or at different vertical positions in the water column (Charrier et al., 2008). There is no published information in the preferred habitats of different *E. siliculosus* strains assessed in this study; however, there is evidence that levels of Chl*a* in phytoplankton can change in the water column (Zakharkov et al., 2012). These authors found that levels of

Chl*a* in phytoplankton increased as deeper in the water column, which might have been related with greater production of Chl*a* as the availability of light decreased. The latter suggests that different Chl*a* and Chl*c* content between the *E. siliculosus* might be related to their original vertical preferred position, in the intertidal or subitdal. There is also evidence showing that Cu exposure can affect photosynthetic pigments in brown algae. For example, Nielsen and Nielsen (2010) found that *Fucus serratus* individuals collected from Cu polluted locations significantly decreased their Chl*a* and Chl*c* content with increasing Cu exposure; Chl*a* levels went approximately from 400 µg g^{-1} dry weight to 140 µg g^{-1} when exposed to 127 µg L^{-1} Cu. On the other hand, Chl*c* went approximately from 250 µg g^{-1} dry weight to 60 µg g^{-1}. Our results showed that levels of Chl*a* and Chl*c* only decreased for the strain LIA4A when exposed to high Cu exposure, but not for Es524 and REP10-11, suggesting that photosynthetic pigments in LIA4A were oxidised by Cu-induced excess of ROS. Another reason can be related to the known Cu-induced inhibition of photosynthesis, recorded before in *E. siliculosus* (Küepper et al., 2002) and in *F. serratus* (Nielsen et al., 2003; Nielsen and Nielsen, 2010). Exposure to Cu can induce photo-inhibition of PSII due to Fe deficiency, which can cause a decrease on the production of chlorophyll (Patsikka et al., 2002). The latter authors observed that Cu outcompetes with Fe in its uptake, making Fe less available in the chloroplast; Fe is an essential metal, and necessary for the biosynthesis of chlorophyll (Fodor et al., 1995). Moreover, Kupper *et al.* (2002) found that photo-inhibition in *E. siliculosus* can be caused by substitution of Mg^{2+} by Cu^{+2} in the chlorophyll molecule and lead to its degradation. The latter authors did not find Chl*c* in Cu stressed *E. siliculosus*; however, they used 500 nM Cu^{2+}, much higher than our highest exposure of 128 nM Cu^{2+} (2.4 µM Cu).

Under photo-oxidative stress, triple state chlorophyll is produced, which can easily react with molecular oxygen to produce the $^1O_2$, a highly oxidizing ROS (Girotti and

Kriska, 2004). In terrestrial plants, it has been found that zeaxanthin, a type of xanthophyll, is capable of quenching $^1O_2$, and act as an antioxidant in events of photo-stress (Dall'Osto et al., 2010). Fucoxanthin is the main xanthophyll and light harvesting pigment present in brown algae, which absorbs in the blue-green to yellow-green part of the visible spectrum, and is responsible for their brown colour (Evstigne and Paramono, 1974). Recently, fucoxanthin has received increased attention due to its strong reducing and antioxidant characteristics, proving significant effects in the treatment of ailments such as cancer and obesity in humans (Masashi and Kazuo, 2007). It has been observed that fucoxanthin can quench $^1O_2$ and •OH *in vitro*, and has higher antioxidant capacity than α-tocopherol (Mikami and Hosokawa, 2013). Moreover, It has been recognized that within the fucoxanthin-chlorophyll a/c-binding protein complexes, this xanthophyll provides photo-protection against triple states of chlorophyll (Di Valentin et al., 2012). Despite the latter, nothing has been described on the importance of fucoxanthin as a ROS scavenger for brown algal cells. Furthermore, apart from the described induction of xanthophylls to counteract the effects of ROS excess due to photo-stress, there is no information on their synthesis against other ROS inducers, such as Cu excess. In our study, we found that for the strain LIA4A, fucoxanthin levels decreased when exposed to high levels of Cu, same as observed in Chla and Chlc. REP10-11 was constant in Chla, Chlc, and fucoxanthin levels. Finally, Es524 maintained Chla and Chlc, but increased levels of fucoxanthin with Cu exposure. This supports the hypothesis that the reason for the deficiency of chlorophyll and fucoxanthin in LIA4A is the Cu-induced oxidative damage, and not Cu-mediated photo-inhibition; Cu exposure might have induced lack of Fe or substituted Mg^{2+} and therefore produced chlorophyll deficiency, but not necessarily affect levels of fucoxanthin. The latter might suggest that fucoxanthin in Es524, in addition to its light harvesting function, is also playing an antioxidant role in the chloroplast, and protecting chlorophyll from ROS damage due to Cu excess.

To counteract the effects of oxidative stress, glutathione is one of the most important cellular antioxidants (Noctor et al., 2012). Glutathione in its reduced form (GSH) is a tripeptide composed of glutamate, cysteine, and glycine. Amongst its many detoxification functions, it is a general reducing agent which is capable of donating protons and electrons to more reactive molecules such as ROS. As in many other organisms, in brown algae, it has been found that metal excess can trigger an increase in the total glutathione pool indicating the activation of the antioxidant machinery (e.g. Contreras et al., 2005; Pawlik-Skowronska et al., 2007; Contreras et al., 2009). In presence of ROS, GSH is oxidized to GSSG, which is then reduced back to GSH in the presence of NADPH by the enzyme GR. Ratios of GSH:GSSG can change between species and specific tissues; however, a decrease in ratio from baseline values have been recognized as reliable markers and commonly used as indicators of oxidative stress (Noctor et al., 2012). In agreement with the literature, levels of total glutathione increased for all the strains assessed with increasing Cu exposure, but relationships between GSSG and GSH ratios where highly strain-specific. Although GSH:GSSG ratios decreased for all strains with higher Cu exposure, LIA4A showed the greatest decline, with values below 1 when exposed to 1.6 and 2.4 µM of Cu. The smallest decrease in ratios was observed in REP10-11, followed by Es524 (although values always above 1). Our results suggest that more GSH is produced and better recycled in the strains REP10-11, followed by Es524. In contrast, the lower levels of total glutathione pool and significant decrease of GSH below GSSG levels in LIA4A, might suggest that there was an intense GSH oxidation to GSSG by ROS, and clear signs redox unbalance due to poor GSH recycling.

Greater levels of reduced ascorbate (Asc) were found with increasing Cu exposure for the strains REP10-11 and Es524, but the latter showed the highest content.

For LIA4A the levels of Asc increased with Cu exposure until 0.8 µM, but then decreased even below levels of control samples. Similar behaviour could be observed with DHA, with greater levels in all strains with increasing Cu exposure. The levels of DHA were clearly higher than Asc for LIA4A at 1.6 and 2.4 µM of Cu, which might suggest a high level of Asc oxidation to MDHA, and finally to DHA. In Es524 and REP10-11, similar patterns could be observed, although in much lesser extent than in LIA4A. Similar observations on DHA increase upon Cu stress have been made in other brown algae species such as *L. nigrescens* and *S. lomentaria* (Contreras et al., 2005; Contreras et al., 2009) and the green alga *Ulva compressa* (Ratkevicius et al., 2003; Mellado et al., 2012). However, as presented in this investigation, this pattern can also change between different populations. From the relationships in the glutathione-ascorbate cycle, a clear pattern can be highlighted. In LIA4A, DHA and GSSG were much higher than GSH and Asc, respectively, when exposed to high Cu concentrations (similar behaviour in Es524 but in lesser extent). In contrast, levels of Asc and DHA were similar for REP10-11 in all experimental treatments, including high Cu exposure. Moreover, the GSH:GSSG ratios confirmed that in REP10-11, GSH levels were always much higher than GSSG. These results suggest that REP10-11 has more GSH available in events of oxidative stress, which in a DHAR catalysed reaction is used as substrate to better recycle Asc. The latter appears to be an important antioxidant mechanism for REP10-11, and more efficient than in the strains Es524 and LIA4A.

Other important molecules used to combat metal stress in algae are phenolic compounds. They are secondary metabolites, which are not directly involved in growth and development of living organisms (Croteau et al., 2000). In addition to their role as chelators to reduce metal concentration extra- and intra-cellularly (Gledhill et al., 1999), and relevance as adhesive for fouling species (Potin and Leblanc, 2006), they are also

important antioxidants. Although there was an increase in phenolic compounds levels with greater Cu exposure in the *E. siliculosus* strains assessed, which is an indicator of antioxidant response, there was no clear pattern between them.

Algae have a battery of enzymes that contribute to the control of ROS. Also called "redox enzymes" or "antioxidant enzymes", some of the most important ones are superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APx) (see Pinto et al., 2003). For instance, high levels of SOD activity have been described in *F. vesiculosus* under the effects of the cyanotoxin nodularin (Pflugmacher et al., 2007) and temperature stress (Collen and Davison, 2001); however, to the extent of our knowledge, SOD activity has not been reported under metal stress in brown seaweeds. The strain Es524 displayed the highest SOD activity when exposed to Cu. On the other hand, REP10-11 showed higher activity than LIA4A. REP10-11 did not change the activity of SOD from baseline levels and LIA4A increased its activity only at the highest levels of Cu exposure; both strains only presented similar SOD levels when exposed to 2.4 µM of Cu. The data showed that the strain Es524 increases the activity of SOD immediately when Cu threat is present, even at low concentrations (0.4 µM of Cu), and maintains it constant with greater Cu levels; Es524 appears to be capable of intensifying SOD activity to levels that allow sufficient protection if Cu exposure, and subsequent $\bullet O_2^-$ production, increases.

The enzyme APx, found in plants and algae, helps in the detoxification of H_2O_2 using ascorbate as a substrate; products of this reaction are DHA and water (Nakano and Asada, 1981). Another H_2O_2 scavenger enzyme is CAT, catalysing its decomposition to water and oxygen (Chelikani et al., 2004). As they have the same ROS as a target, it has been found that they both cooperate in the defence against H_2O_2 in organisms such as plants (Asada, 1992; Mizuno et al., 1998) and also in brown algae (Collen and Davison,

2001). The importance of either APx or CAT can vary under different environmental conditions, and is also species-specific (Smirnoff, 2005a; Cansev et al., 2011). Although there was evidence that the activity of APx was greater under increasing Cu concentrations, there was no a systematic trend that allowed differentiation between the *E. siliculosus* strains assessed in this study. Conversely, the activity of CAT was clearly higher in the strain Es524 and, moreover, it was the only strain where the CAT activity increased in parallel with the increasing levels of Cu exposure; the activity of CAT in either LIA4A or REP10-11 did not seem to be affected by Cu concentrations. Contreras et al. (2009) reported results on APx and CAT in *Lessonia nigrescens* and *Scytosiphon lomentaria* under Cu stress; in both species, the enzymes APx and CAT cooperated and increased their activity with growing Cu exposure, however, APx and CAT activities were much higher in *L. nigrescens* than in *S. lomentaria*. Contreras et al. (2009) collected both *L. nigrescens* and *S. lomentaria* individuals from locations with no pollution history, therefore, very likely to correspond to 'non-tolerant populations'. In our study, only the strain Es524 was capable of increasing both CAT and APx activities under Cu stress. In case of the strains LIA4A and REP10-11, defence against H_2O_2 appeared mainly to depend on the action of APx, and to lesser extent CAT. The activities of CAT and APx are in agreement with the results on H_2O_2 content. The data suggests that CAT and APx in Es524 cooperate in the action against H_2O_2, maintaining redox equilibrium. In LIA4A, the only increasing activity of APx under Cu exposure does not seem to be sufficient, and therefore H_2O_2 is much higher than in the other strains. In REP10-11, although only APx activity increased with Cu exposure, there was a small increase in H_2O_2 at the highest Cu exposure; these results might suggest that the action against H_2O_2 in REP10-11 is related, rather than strictly to CAT and APx, to other peroxidase enzymes. This is the first investigation reporting that the collaboration in the action between APx and CAT can be strain- or population-specific in brown algae, and not only species-specific.

There is evidence that brown seaweed populations under different levels of metal stress can develop tolerant ecotypes. For example, Nielsen et al. (2003) found in the laboratory that a population of *F. serratus* from a Cu polluted location had better embryo and adult development under Cu stress than a population coming from a pristine site. Pawlik-Skowronska et al. (2007) found higher metal accumulation, greater production and longer chain variants of the metal chelators phytochelatins, and greater glutathione content in *Fucus* spp. sampled in locations with high metal pollution history (including Cu), in comparison to pristine sites. Now, that *E. siliculosus* has been positioned as a model organism (Peters et al., 2004a) and that several different strains have been collected from locations around the world and isolated for their study (Dittami, Simon M. et al., 2011), new opportunities lie ahead for the study of brown algae. To date, only one investigation has shown signs of development of ecotypes to Cu stress in *E. siliculosus*. Ritter et al. (2010) performed Cu exposure experiments on two strains, Es524 (also included in our investigation) and the strain Es32 (from San Juan de Marcona, Peru). For example, they observed that the gene encoding the manganese-stabilizing protein of the oxygen evolving complex of photosystem II (*OEC33*) was more expressed in Es524 than in Es32; they suggested that this upregulation could result in better recycling of OEC33 damaged by ROS in Es524. Although the latter might indicate development of ecotypes in different strains of *E. siliculosus* to Cu-mediated oxidative damage, no empirical data confirmed these hypotheses. Oxidative stress responses in the three *E. siliculosus* strains assessed in this investigation revealed the existence of ecotypes in Cu resistance, which in levels of resistance can be arranged in descent as: Es524 > REP10-11 > LIA4A (see explanatory table 3.2). Interestingly, even though both Es524 and REP10-11 are originally from locations of high Cu pollution history, their antioxidant strategies to address Cu stress are clearly different. It is important to point out that the location from where REP10-11 was

collected (Restronguet Creek, England), has a metal pollution history (Cu and other metals) dating from middle 19th century, when Cu miners started to establish; there are older records of mining activity in the area, however, only micro-scale and not at industrial level (Somerfield et al., 1994). In contrast, Es524 was collected from a location where Cu mining and consequent pollution has a history dating from the 16th century (Chañaral, Chile) (Maksaev et al., 2007); Cu mining in northern Chile is a result of the high content of this metal in the Atacama Desert (Maksaev et al., 2007). Moreover, there is evidence that mineralization in the middle Miocene and subsequent erosion in the Atacama Desert could have facilitated sediment and Cu transportation to coastal areas (Alpers and Brimhal, 1988). Therefore, high Cu resistance in Es524 can be related to a long history of Cu mining pollution, but also to Cu enrichment in the coastline of northern Chile dating from ancient times.

Table 4.2. Summary of Cu resistance in the three *Ectocarpus silicuosus* assessed. Es524 and REP10-11 are originally from locations with history of Cu pollution in Chile and England, respectively; the strain LIA4A is from Scotland, a pristine site. More + symbols indicate that the strain was better able to cope with Cu-induced oxidative stress based on the each measurement conducted. For example, strain Es524 showed low levels of H_2O_2, but high levels of catalase. Cu resistance was ranked as: Es524 > REP10-11 > LIA4A.

Oxidative stress indicators	**Es524**	**LIA4A**	**REP10-11**
H_2O_2 levels	+++	+	++
Lipid peroxidation	+++	++	+
Oxidation of pigments	+++	++	+++
Antioxidants			
Glutathione	++	+	+++
Ascorbate	+++	+	+++
Phenolic compounds	++	+	++
Fucoxanthin as antioxidant	+++	+	+
Activity of antioxidant enzymes			
Superoxide dismutase	+++	++	++
Ascorbate peroxidase	+++	+++	+++
Catalase	+++	+	+

Genomic and epigenetic adaptations could have an important role in the divergent responses to metal stress. For example, although there is no available information for brown algae, Chang & Leu (2011) found that a diploid strain of yeast from a Cd polluted site, and the only among others able to grown under 0.8 mM $CdCl_2$, had three nucleotide mutation in the promoter sequence of the *PCA1*, gene encoding a P-type ATPase enzyme

required for cadmium efflux (Shiraishi et al., 2000). Chang & Leu (2011) observed that this mutation was critical and permitted a significant over expression of *PCA1* compared to other strains when Cd threat was present. More research in this direction will help elucidating the importance of genomic and epigenetic adaptations to counteract the effects of metal excess in brown algae, including oxidative stress.

Our study revealed that three strains of *E. siliculosus* assessed had different antioxidant responses under Cu stress, Es524 being the most efficient ecotype in regard to its antioxidant defences, and the strain LIA4A the most sensitive. There are records showing brown algae metal resistance in the literature; however, the mechanistic and metabolic reasons behind their resistance have remained elusive. This study has demonstrating that brown algae are capable of displaying a broad set of antioxidant defences to counteract the effects of Cu-mediated oxidative stress and, moreover, showed that Cu resistance by means of antioxidant defences in brown seaweeds is not only species-specific, but it can also be population-specific. Our results suggest that inherited factors related to the exposure history of the strains assessed may have played a relevant role in this process. Due to the popularity of brown seaweeds for metal biomonitoring purposes, the information gathered in this investigation must be also taken into account when using oxidative stress biomarkers to assess metal pollution in the field, as the divergent inter-populations responses can lead to false environmental diagnosis.

Chapter V

In situ assessment of metal pollution using oxidative stress responses in the model brown alga *Ectocarpus siliculosus*

5.1. Introduction

There is growing concern that the natural cycling rates of many metals are being disrupted or accelerated as a consequence of anthropogenic activities which are threatening marine life and potentially human health (Bruland and Lohan, 2006). Many metals, including Fe, Cu, Zn, and Mg, are essential for metabolic processes in chloroxygenic organisms (Falkowski and Raven, 2007; Yilmaz et al., 2010), whereas others such as Cd, Pb, and Al, are non-essential and can be toxic at trace levels (Brown and Depledge, 1998). Beyond certain threshold concentrations, essential and non-essential metals can produce detrimental effects on marine algae, such as photo-inhibition, disruption of electron transport chains in chloroplasts and mitochondria, causing oxidative stress by accumulation of reactive oxygen species (ROS) (Torres et al., 2008). For example, high concentrations of Cu exposure can affect the redox potential of NADPH-dependent oxidases producing an electron transfer to oxygen and the subsequent over-production of $\bullet O_2^-$, which is precursor of H_2O_2, a less reactive ROS (Gonzalez et al., 2010). In order to counteract the effects of metals and subsequent ROS excess, marine algae have developed a strong defence machinery, based on extracellular exclusion mechanisms associated with metal chelation in the cell wall, synthesis of metal-chelating molecules, the production of antioxidant compounds such as glutathione and ascorbate, and the activation of antioxidant enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase (Pinto et al., 2003; Torres et al., 2008; Gonzalez et al., 2010). The vast majority of the research conducted in relation to the biological defences of algae against metal stress has been obtained in laboratory experiments; however, there is a lack of similar evidence under field experiments, which can compromise extrapolation of research to the natural, more complex, environment.

In order to assess the reach of the impacts associated with metal pollution in marine ecosystems, different methods have been developed to identify their presence in the environment, and their concentrations and effects on marine biota. One possibility is to determine metal concentrations in sediments and water; this methodology is useful to discriminate and identify environmental fluctuations but does not provide information about biological effects (Brown and Depledge, 1998). A more representative approach is biomonitoring, which uses biological stress responses at different levels of organization for environmental diagnosis. While it is widely recognised that metal pollution induces stress-mediated changes at population, community, and ecosystem levels, these higher level changes are typically too complex and far removed from the causative events to be practically used for the early detection and prediction of the consequences of anthropogenic activity (Gonzalez et al., 2010). Conversely, environmental stress can be studied by assessing biochemical signals which act as early warning biomarkers of reduced individual health and fitness (Brown and Depledge, 1998; Torres et al., 2008). Metal excess can produce rapid oxidative stress, which can result in cell injury, molecular damage and impairment of protective systems as well as reduction in reproductive output, competitive ability and survival of individuals (Brown and Depledge, 1998; Torres et al., 2008).

Brown seaweeds are the main primary producers at the base of trophic networks, providing habitat for a large diversity of organisms in coastal and estuarine ecosystems (Smith, 1996b; Sáez et al., 2012a; Sáez et al., 2012b). Their ecological importance, metal accumulation capacity and ability to survive in highly metal polluted sites have made brown seaweeds prime candidates as biomonitors for environmental assessment of metal pollution (Pinto et al., 2003; Sáez et al., 2012a). Metal assessments using brown algae and other species (e.g. Ulvophyceae) have usually been conducted with a ‘passive-

biomonitoring' approach, where native organisms of the ecosystems are sampled and metal concentrations measured (e.g. Ratkevicius et al., 2003; Pawlik-Skowronska et al., 2007; Sáez et al., 2012a). However, some algae are known to develop resistance after prolonged exposure to metals, a trait that can be inherited and result in resistant ecotypes (Ritter et al., 2010; Trzcinska and Pawlik-Skowronska, 2013), and as could be observed on our results in Chapter III and IV of this thesis, on different strains of *Ectocarpus siliculosus* under Cu exposure experiments in the laboratory. Differences in metal accumulation and stress responses between population of the same species can bring uncertainties when conducting biomonitoring and lead to false environmental diagnosis (Brown et al., 2012). Several authors have suggested that 'active-biomonitoring' could be a better, alternative option; organisms are cultured in controlled laboratory conditions and their responses to metal exposure assessed before being introduced into sites of interest (Chaphekar, 1991; Brown et al., 2012; Sáez et al., 2012a). Advantages of such an approach include the capacity to perform: the transplantation of individualsfrom one or more populations into different locations, use of individuals of similar, and a range of, age(s), and assessing pollution levels of locations from where the biomonitor is absent. Active-biomonitoring with brown algae has mostly been with kelps that were reciprocally transplanted between sites (Serisawa et al., 2002; Hédouin et al., 2008). However, transplantation of these organisms is both logistically complex and can be time-consuming due to their large size and relatively slow growth. Moreover, metal accumulation varies between different thallus parts of complex brown seaweeds, which is likely to be related to changes in cell wall composition such as alginate concentration and composition (Sáez et al., 2012a). Therefore, for these reasons, using morphologically less complex brown algal species are likely to be more amenable for active-biomonitoring.

Ectocarpus siliculosus is a small filamentous brown alga that inhabits substrates from the low intertidal to the shallow subtidal, buoyant or attached to hard substrate or as an epiphyte of larger seaweeds (Charrier et al., 2008). The species has a global distribution in coastal temperate environments, and has been recently recognized as a model organism for brown algae (Peters et al., 2004a; Coelho et al., 2012b). Several characteristics highlight *E. siliculosus* as a good model organism: it can be easily cultured in the laboratory, it has a small stature and fast growth, it is anatomically and physiologically less complex than kelps and fucoids, but is closely related to them (Charrier et al., 2008). Several strains coming from locations around the world have been collected and isolated for their study (Dittami, S. M. et al., 2011), providing the opportunity to study inter-population differences in response to environmental stressors such as metals, and to use this information to improve our current biomonitoring protocols. To this end, a novel active-biomonitoring method to determine metal pollution through transplantation experiments using different strains of *E. siliculosus* has been developed. Based on the results obtained from the Cu exposure experiments exposed beforehand in this thesis, different parameters were assessed, related to metal accumulation and reactive oxygen metabolism; more specifically, lipid peroxidation and concentrations of H_2O_2, pigments chlorophylls *a* and *c*, and fucoxanthin, antioxidants glutathione and ascorbate in reduced and oxidised forms, total phenolic compounds, and the antioxidant enzymes superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase. The aims of our investigation were: to assess the efficiency of the transplantation method for pollution environmental diagnosis; to identify mechanistic differences in stress responses between strains of *E. siliculosus in situ*; and to discuss the implications of using seaweed biomarkers for biomonitoring metal pollution in estuarine and coastal waters.

5.2. Materials and methods

5.2.1. Locations for transplantation experiments

Two different locations in Chile were chosen for the transplantation experiments, one polluted and one pristine. From available data (Gonzalez et al., 2008; Sáez et al., 2012a; Sáez et al., 2012b), the bay of Ventanas in Central Chile (32°44'36.55"S and 71°29'35.70"W) was chosen as the contaminated site (Figure 5.1). Sources of pollution come from Cu melting and casting industries and thermoelectric complexes (Neaman et al., 2009), and from wastewaters with unknown origin delivered by an illegal sewage outfall (Sáez et al., 2012a; Sáez et al., 2012b). The pristine location assessed was Quintay (33°11'46.16"S and 71°42'18.73"W) (Fig. 1), a site with no history of metal contamination.

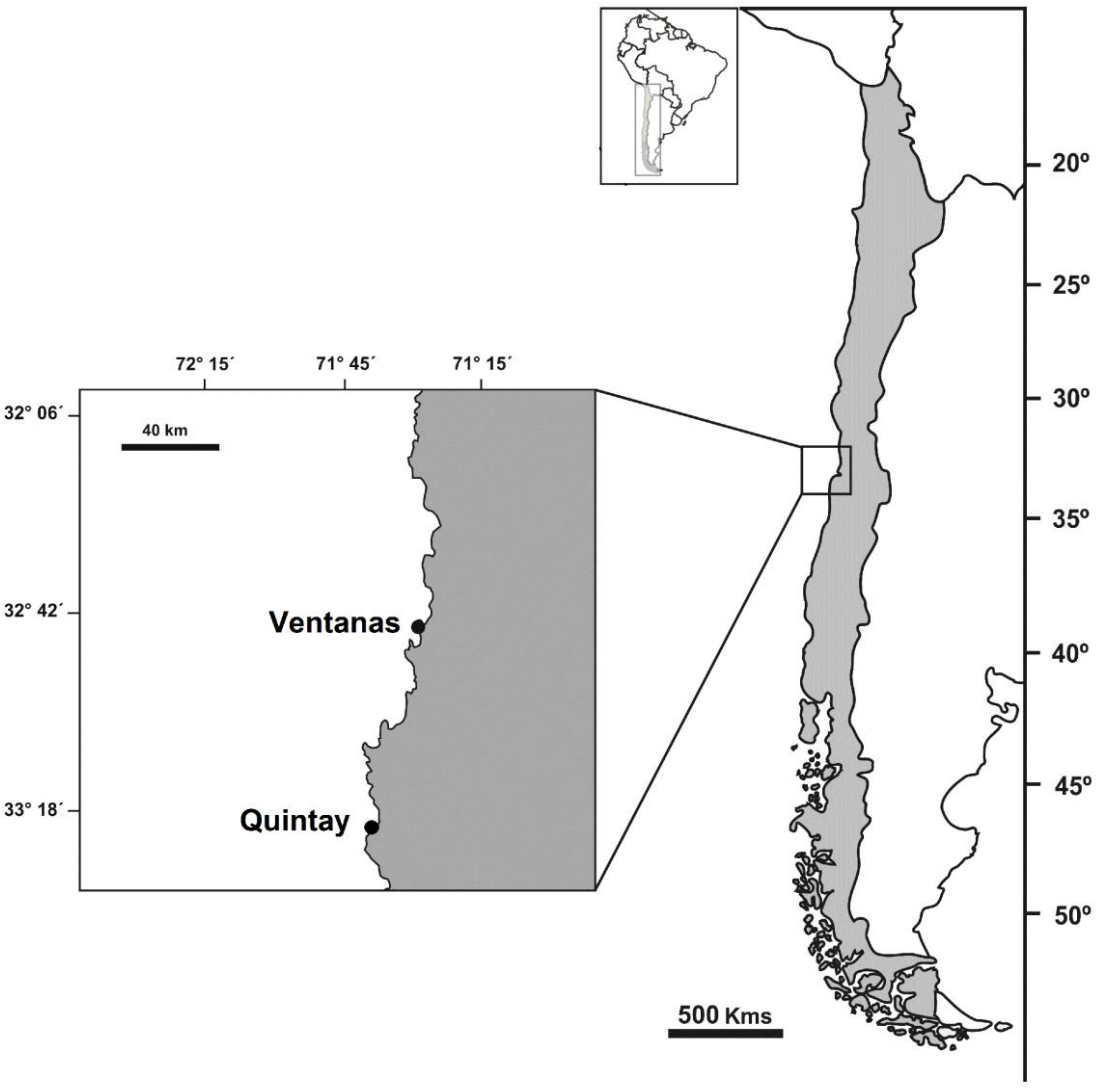

Figure 5.1. Map of the locations for transplantation experiments in Central Chile.

5.2.2. *E. siliculosus* strains and culture

The strains were obtained from the Station Biologique Roscoff, France, and the Marine Biological Association of the United Kingdom. Es524 strain was isolated from Caleta Palito (Chañaral, Chile), a coastal site with a long history of Cu pollution from mining activities; this strain was also used in the laboratory experiments in Chapters III and IV. Strain Es147 was from Caleta Coloso (Antofagasta, Chile), a harbour with recent history of low metal inputs. To avoid strain-crossing and proliferation of non-native *E. siliculosus* in the locations assessed, strains native to Chile were used. The strains were cultured in 10 L polycarbonate bottles with autoclaved seawater and Provasoli nutrients (Provasoli and Carlucci, 1974), at 16ºC, 45 µmol photons m^2 sec^{-1} on 14:10 light:dark cycle, and bubbled with sterile air to avoid CO_2 depletion and maintain material in suspension.

5.2.3. Transplantation device and field experiments

Ectocarpus siliculosus is a small seaweed that grows up to 30 cm (Charrier et al., 2008); transplantation efforts without an enclosure would inevitably lead to loss of biomass and biological contamination (i.e.. bacteria, fouling algae). Therefore, seaweed samples were enclosed in dialysis tubing cellulose membranes permeable only to small molecules and ions including metals (De Philippis et al., 2003); experiments were conducted in triplicates. For transplantation purposes, 76 mm flat width dialysis tubing with 14,000 g mol^{-1} molecular mass cut-off (Sigma-Aldrich, D9402) was used, and the tubing filled with autoclaved seawater as used for culturing. Because dialysis tubing is made of cellulose and might be edible to herbivores, the tubing was placed inside a transparent 2 L plastic bottle with a hundred 1 mm holes to facilitate water exchange. The

device was deployed, attached to a c. 5 kg rock with nylon fishing line, and placed 2 m below the lowest tide (Figure 5.2). The transplants were collected 10 days after deployment. At the end of the exposure period seaweed samples were immediately blotted dried and frozen in liquid nitrogen, transported to the laboratory and stored at -80ºC for further analyses.

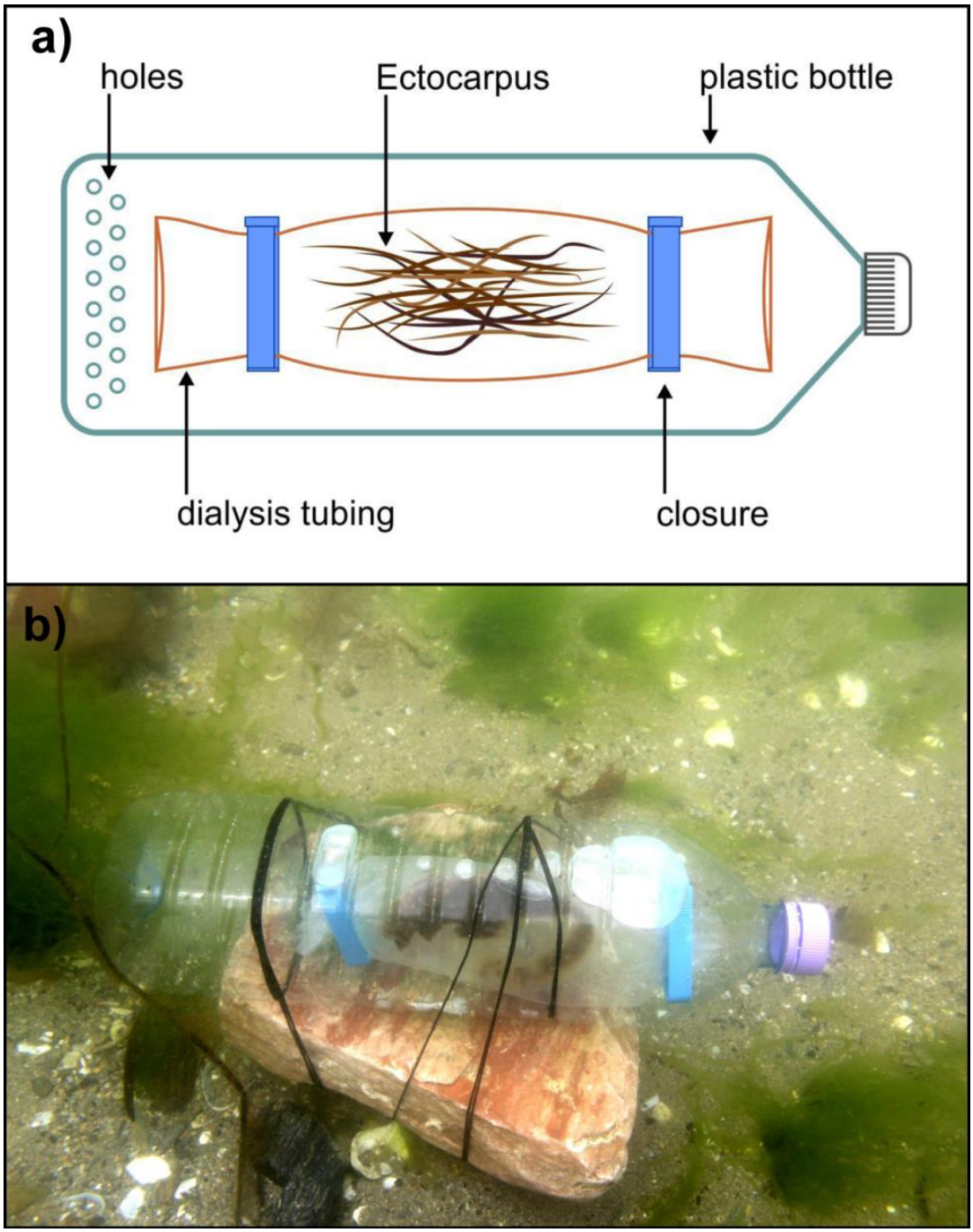

Figure 5.2. Transplantation device: a) Diagram of the device b) Sample of the device in the field .

5.2.4. Metal accumulation

E. siliculosus biomass (40 mg) was freeze-dried for 24 h, and then digested with 2 mL of HNO_3 in a MARS 6 microwave (Table 5.1). After digestion, the volume of each digest was adjusted to 10 mL with milli-Q water (18 Ω). The total concentration of Cu, Cd, Al, Fe and Pb were determined using ICP-MS (Thermo Scientific, X Series 2). The choice of metals was based on a recent study that found high concentrations in sediments and in the kelp *Lessonia trabeculata* in Ventanas (Sáez et al., 2012a). To validate the methodology, the digestion was also applied to reference material (*Ulva lactuca*, BCR-279) (Table 5.2).

Table 5.1. Microwave cycles for metal measurement with algal acid digestion

Maximum	**Ramp time**	**°C**	**Hold time**
1600	5:00	120	2:00
1600	5:00	155	2:00
1600	5:00	170	15:00
Cool down			10:00

Table 5.2. Metal in certified material BCR-279 (sea lettuce, *Ulva lactuca*). Errors are ± 1 SD, n = 3

	Al	**Fe**	**Cu**	**Cd**	**Pb**
Found value (mg Kg^{-1})	2.2 ± 0.3 (x 10^3)	2.1 ± 0.3 (x 10^3)	13.5 ± 0.3	0.24 ± 0.03	14.0 ± 0.4
Certified value (mg Kg^{-})	1.90 ± 0.05[a] (x 10^3)	2.4 ± 0.1[a] (x 10^3)	13.1 ± 0.4	0.27 ± 0.02	13.5 ± 0.4

[a] Indicative Values

5.2.5. Measurement of oxidative stress parameters

The concentration of H_2O_2 was measured according to Sergiev et al. (1997) in a plate reader as described in Section 3.2.3. Levels of lipid peroxidation were measured according to Heath and Packer (1968) in a plate reader as detailed in Section 3.2.3.

Concentrations of the pigments chlorophyll *a* (Chl*a*) and *c* (Chl*c*), and fucoxanthin (fx) were measured according to Seely et al. (1972), as described in Section 3.2.3.

5.2.6. Antioxidant compounds

Glutathione in its reduced (GSH) and oxidised forms (GSSG) were measured according to Queval and Noctor (2007), with some modifications for a plate reader as further detailed in Section 3.2.4 of the thesis.

The concentrations of reduced ascorbate (Asc) and dehydroascorbate (DHA) were measured according to Benzie and Strain Benzie and Strain (1999) with a plate reader (see Section 3.2.4).

Concentrations of total phenolic compounds were measured according to Van-Alstyne (1995) as described in Section 3.2.4.

5.2.7. Protein extracts to measure activity of antioxidant enzymes

The extraction procedure to measure the activity of the antioxidant enzymes was changed from that used in section 3.2.5 of this Thesis. We observed that with the former extraction method the activities of antioxidant enzymes in the extract were small, which presented difficulties during the measurement, such as using big volumes of extract and noise during spectrophotometric determination. To overcome this issue, during our stay in Chile, we learnt and applied the method developed by the Laboratory of Molecular Biology of Universidad de Santiago de Chile, published in Ratkevicius et al. (2003). The method is based on the preparation of a concentrated protein extract through precipitation with ammonium sulphate. With the new protocol the activity of antioxidant enzymes was stronger and the noise was decreased; moreover, due to the concentration of proteins, we were able to measure the activity of glutathione reductase, which was not detectable with the former extraction protocol adapted from Collen and Davison (1999) (Section 3.2.5).

Between 5 and 10 g of frozen biomass were ground to powder in a mortar with liquid nitrogen. A solution of 100 mM potassium phosphate buffer (pH 7) containing 5mM 2-mercaptoethanol was added in a ratio of 1g:3mL. The mixture was filtered through

Miracloth paper (Calbiochem) and centrifuged at 21,000 g for 10 min at 4ºC. To precipitate proteins, the supernatant was transferred to a new tube and 0.5 g per milliliter of ammonium sulphate was added; the mixture was vortexed at 400 rpm for 2h at 4ºC. The mixture was centrifuged at 21,000 g for 30 min at 4ºC, and the pellet was re-suspended in 100 mM potassium phosphate buffer (pH 7), containing 2mM 2-mercaptoethanol and 10% glycerol. Protein extracts were adjusted to a final concentration of 1mg mL^{-1} using the Bradford method with bovine serum albumin as standard (Bradford, 1976). Extracts were stored at -80ºC for further enzymatic activity analyses.

5.2.8. Activities of antioxidant enzymes.

Catalase (CAT) was calculated according to Aebi (1984). The activity was quantified by adding 15 µg of protein extracts to 1 mL of 100 mM potassium phosphate buffer (pH 7) and 16 mM H_2O_2. The decrease in absorbance was followed at 240 nm for 30 s and the activity then calculated using the extinction coefficient 43.1 M cm^{-1}. Ascorbate peroxidase (APx) was calculated according to Nakano and Asada (1981). Proteins (15 µg) were added to 100 mM potassium phosphate buffer (pH 7), containing 0.5 mM Asc and 16mM H_2O_2. The decrease in absorbance at 290 nm was monitored for 30 s and the activity was calculated with the extinction coefficient of Asc (ε= 2.8 mM cm^{-1}).

The activity of glutathione reductase (GR) was measured according to Sen Gupta et al. (1993) but with the use a plate reader. Proteins (50 µg) were added to 290 µL of mixture containing 100 mM potassium phosphate buffer (pH 7), 0.5m M oxidized glutathione and 0.15mM NADPH. The decrease in absorbance was followed at 340 nm for 5 min and the activity quantified with the extinction coefficient of NADPH (ε= 6.22 mM

cm^{-1}). The activity of superoxide dismutase (SOD) was quantified in a plate reader, with modifications to that of Mishra et al. (1993). Protein (20 µg) were added to 290 µL of a mixture containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 11 µM cytochrome-c, 11 µM xanthine, and 0.002 Units of xanthine oxidase. Activity of SOD was calculated according to Kuthan et al. (1986).

5.2.9. Statistical analyses

Analyses of Shapiro-Wilk and Bartlett Test were performed to assess requirements of normality and homogeneity of variances, respectively. Differences between mean values of most measured parameters were determined by one-way ANOVA and post-hoc Tukey test at 95% confidence. For Asc and chlorophyll pigments a multivariate ANOVA (MANOVA) at 95% confidence interval was carried out using strains and locations as main factors. The dependent variables were Asc, DHA, Chl*a* and Chl*c*.

5.3. Results

5.3.1. Metal accumulation

The total concentrations of Cu, Fe, Al, Cd, and Pb in both Es524 and Es127 were significantly higher in material transplanted to the polluted site of Ventanas, compare to the pristine location of Quintay ($p < 0.05$) (Table 5.3). Trends showed that the majority of the metals were higher in Ventanas than in Quintay, for both strains ($p < 0.05$); excepting Cd which was higher for both strains in Quintay ($p < 0.05$). When there were significant

differences between strains in each location assessed ($p < 0.05$), Es147 was always the one with the lowest levels; as observed for Cu in Quintay, Fe in Ventanas, Al in Quintay, and Pb in Ventanas.

Table 5.3. Concentrations of metals in the strains of *Ectocarpus siliculosus* Es524 and Es127 transplanted to Quintay (pristine site) and Ventanas (polluted site), located in Chile. Metals Cu, Fe, and Al are expressed in µmol g^{-1} dry weight (DW); Cd and Pb are in nmol g^{-1} DW. Different letters represent significant differences within each metal measured ($p < 0.05$). Errors are ± 1 SD, $n = 3$.

Strain	Location	Cu	Fe	Al	Pb	Cd
Es524	Quintay	1.12 ± 0.11 ^{a}	1.05 ± 0.11 ^{a}	0.7 ± 0.07 ^{a}	10.1 ± 1.4 ^{a}	16.6 ± 2.7 ^{a}
Es147	Quintay	0.74 ± 0.11 ^{b}	0.78 ± 0.09 ^{a}	0.4 ± 0.05 ^{b}	6.1 ± 0.6 ^{b}	14.8 ± 2.1 ^{a}
Es524	Ventanas	9.3 ± 0.8 ^{c}	5.8 ± 0.8 ^{b}	9.6 ± 0.5 ^{c}	78 ± 8 ^{c}	8.3 ± 1.0 ^{b}
Es147	Ventanas	8.1 ± 0.4 ^{c}	4.4 ± 0.4 ^{c}	8.4 ± 0.5 ^{c}	52 ± 3 ^{d}	6.5 ± 1.0 ^{b}

5.3.2. Indicators of oxidative stress

The concentrations of H_2O_2 in Es147 from Ventanas was significantly higher than in Es524 ($p < 0.05$), and also higher than both strains in Quintay ($p < 0.05$) (Figure 5.3a). There were no significant differences between strains in Quintay or with Es524 from Ventanas ($p > 0.05$). The levels of lipid peroxidation followed a similar pattern to H_2O_2 concentrations, as shown in Figure 5.3b.

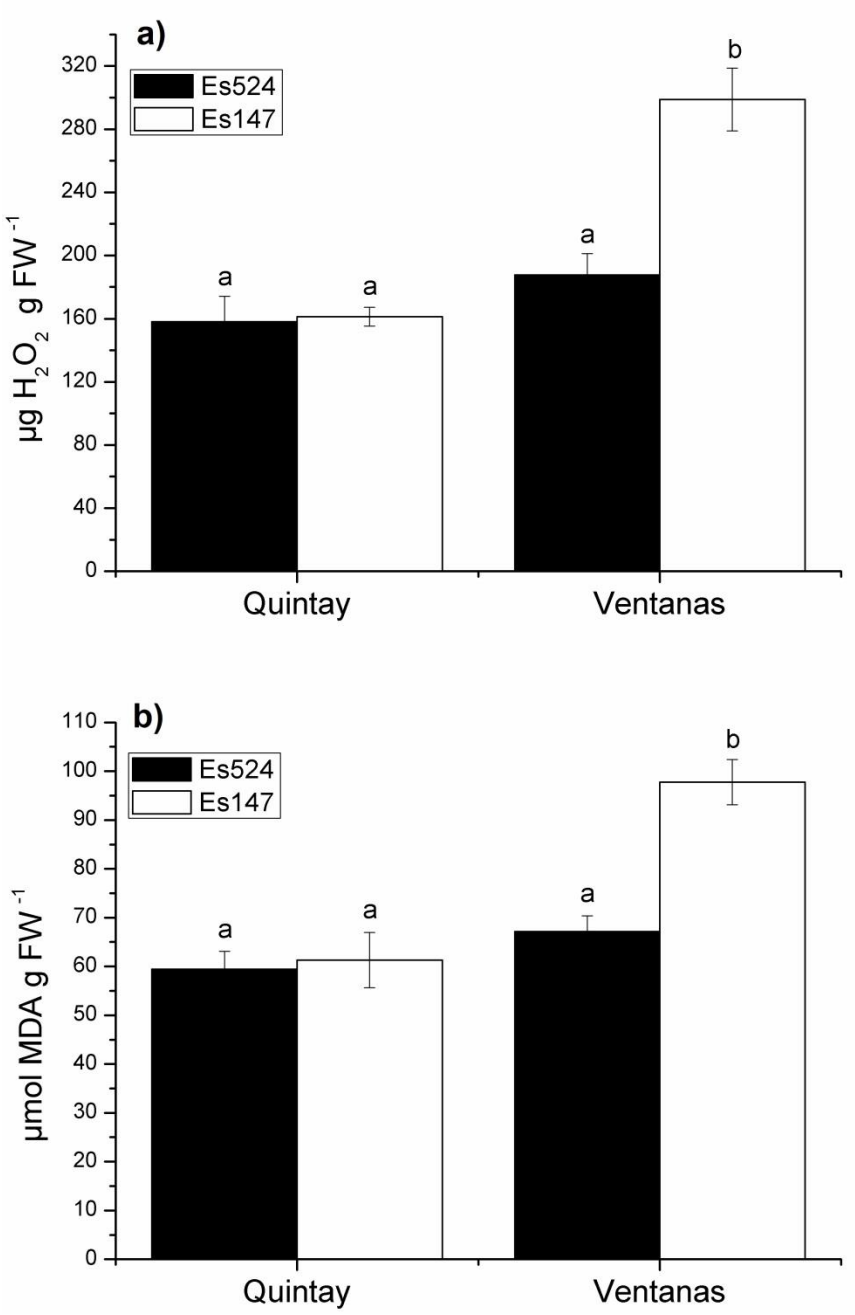

Figure 5.3. Oxidative stress responses in fresh biomass (FW) of two *Ectocarpus siliculosus* strains following transplantation experiments in Ventanas (metal polluted) and Quintay (pristine), in central Chile: a) Hydrogen peroxide concentrations; b) Lipid peroxidation levels; Different letters in bars represent significant differences at the 95% confidence interval ($p < 0.05$). Error bars are ± 1 SD, n = 3.

Significant differences in chlorophyll *a* and *c* (Chl*a* and Chl*c*, respectively) concentrations were observed between strains and locations, and there was a significant interaction between these factors (Pillai's Trace, $p < 0.05$). Concentrations of Chl *a* were consistently significantly higher than those of Chl *c*. The highest concentrations of Chl *a* and *c* were found in Es147 grown in Quintay and the lowest concentrations were found in Es147 from Ventanas ($p < 0.05$) (Figure 5.3a). The highest concentrations of fucoxanthin were found in Es524 from Ventanas, with values significantly higher than in all other material Ventanas ($p < 0.05$); there were no significant differences between Es524 and Es147 from Quintay and Es147 from Ventanas ($p > 0.05$) (Figure 5.3b).

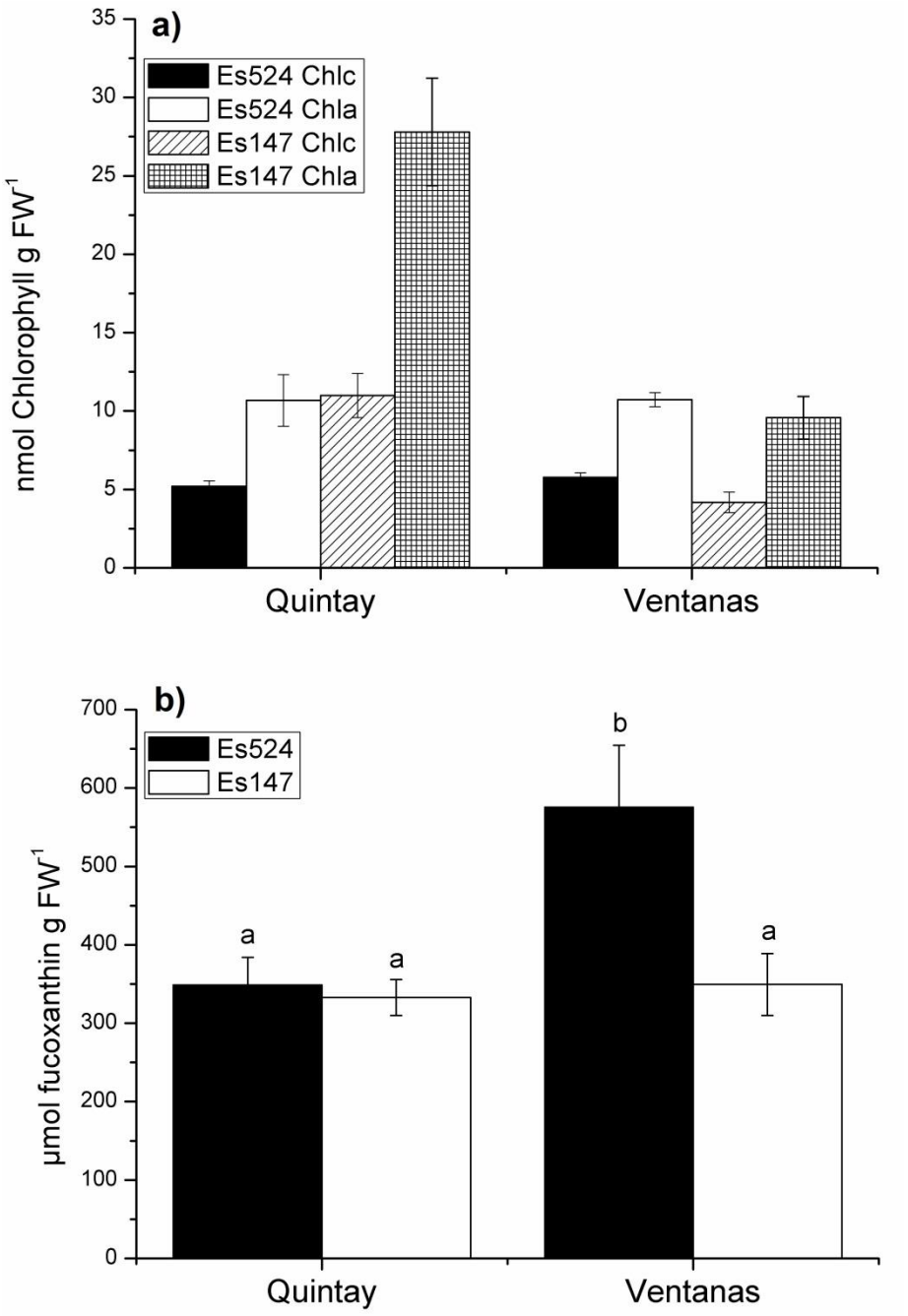

Figure 5.4. Oxidative stress responses in fresh biomass (FW) of two *Ectocarpus siliculosus* strains following transplantation in Ventanas (metal polluted) and Quintay (pristine), in central Chile for 10 days: a) Chlorophyll *a* (Chl*a*) and *c* (Chl*c*); b) Fucoxanthin concentrations. Different letters in bars represent significant differences at the 95% confidence interval ($p < 0.05$). Error bars are ± 1 SD, n = 3.

5.3.3 Antioxidant compounds

Concentrations of total glutathione in the two strains grown in Quintay did not differ significantly ($p = 0.72$), but were significantly lower than in material from Ventanas ($p < 0.05$) (Figure 5.5). At Ventanas, concentrations of total glutathione in Es147 were significantly higher than in Es524 ($p < 0.05$). Concentrations of GSH were similar for the two strains at both sites ($p > 0.05$). The significantly lower GSH/GSSG ratios at Ventanas compared with Quintay reflect the increased concentrations of oxidized glutathione in the strains grown in Ventanas (Fig. 5.5; Table 5.4).

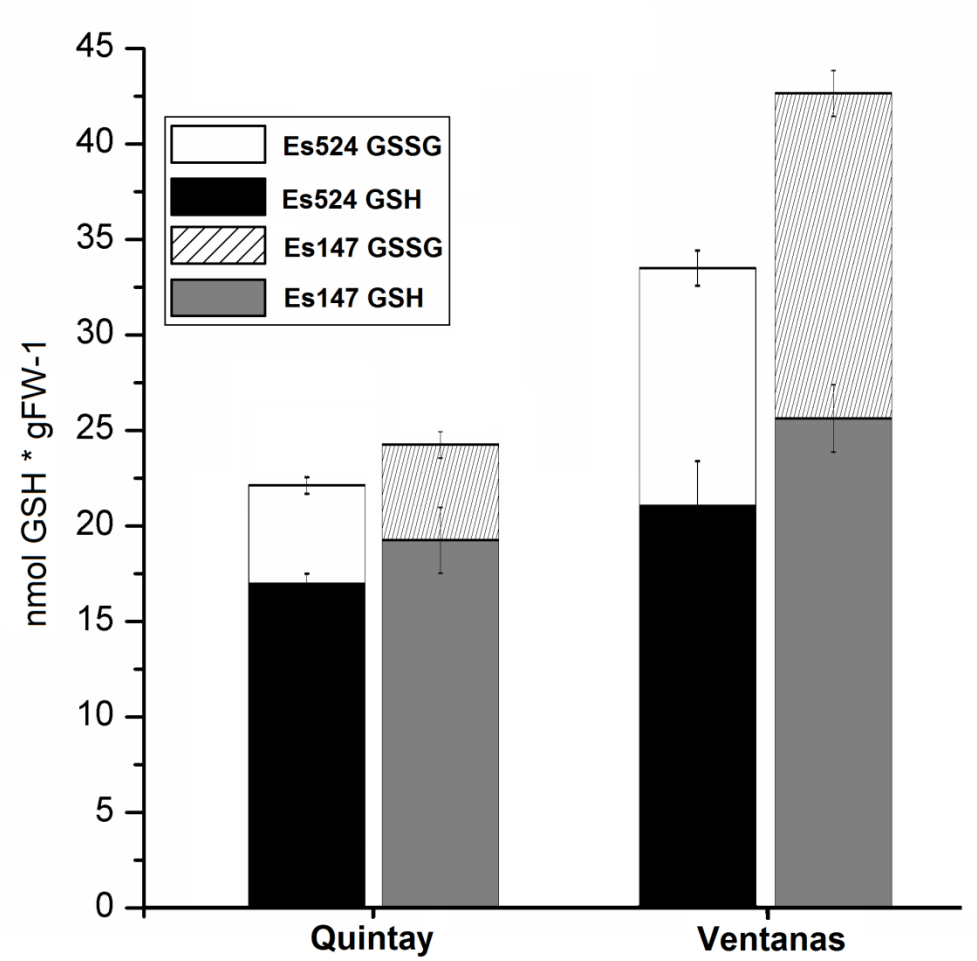

Figure 5.5. Glutathione in reduced (GSH) and oxidised (GSSG) forms in two *Ectocarpus siliculosus* strains following transplantation experiments in Ventanas (metal polluted) and Quintay (pristine), in central Chile for 10 days. Error bars are ± 1 SD, n = 3.

Table 5.4. Ratios between reduced and oxidised glutathione (GSH:GSSG) of the *Ectocarpus siliculosus* strains transplanted in Ventanas (metal polluted) and Quintay (pristine), in central Chile for 10 days. Different letters represent significant differences ($p < 0.05$). Errors are ± 1 SD, $n = 3$

Strain	Location	Ratio GSH:GSSG
Es524	Quintay	3.32 ± 0.36 [a]
Es147	Quintay	3.90 ± 0.55 [a]
Es524	Ventanas	1.70 ± 0.31 [b]
Es147	Ventanas	1.51 ± 0.12 [b]

The concentrations of both Asc and DHA were significantly higher in Es524 and Es147 from Ventanas than Quintay ($p < 0.05$); this was most notable for DHA with a 10 fold increase (Figure 5.6). Concentrations of Asc were consistently higher in Es524 than in Es147 ($p < 0.05$), whereas there were no significant differences between strains for DHA ($p > 0.05$).

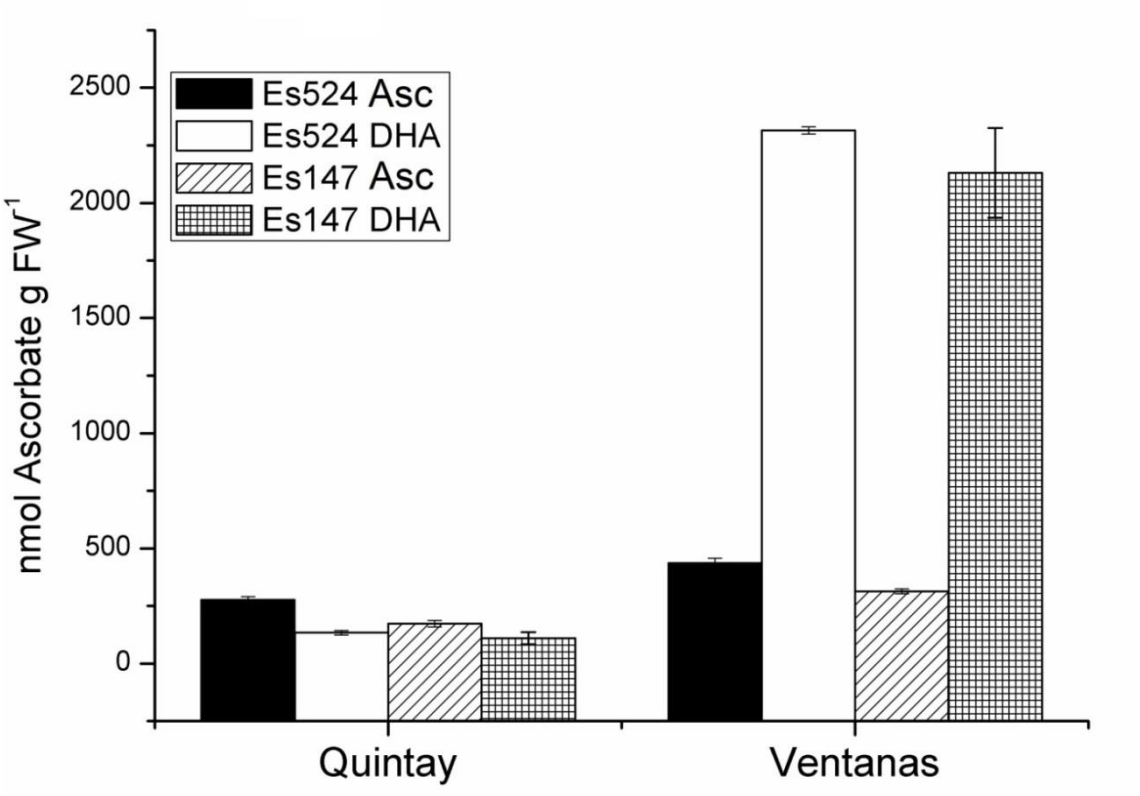

Figure 5.6. Ascorbate in reduced (Asc) and oxidised (DHA) forms in two *Ectocarpus siliculosus* strains following transplantation experiments in Ventanas (metal polluted) and Quintay (pristine), in central Chile for 10 days. Error bars are ± 1 SD, n = 3.

The highest concentrations of phenolics were in Es147 from Ventanas ($p < 0.05$), not significantly different between Es524 and Es147 in Quintay ($p > 0.05$). Concentrations of phenolics in Es524 from Ventanas were significantly higher than from Quintay ($p < 0.05$), but there were no significant differences between strains grown in Quintay or between Es524 from Ventanas and Es147 from Quintay ($p > 0.05$) (Figure 5.7).

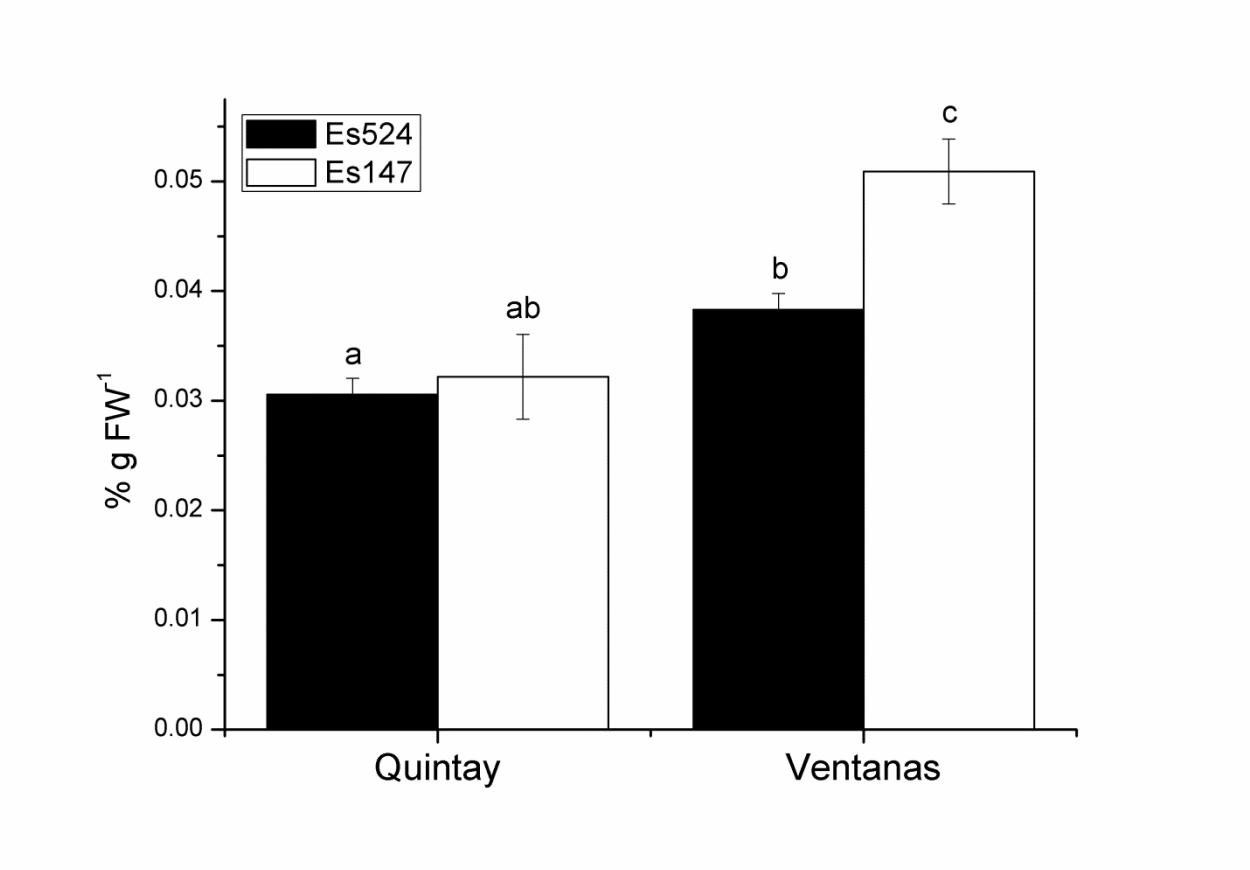

Figure 5.7. Concentrations total phenolics in two *Ectocarpus siliculosus* strains following transplantation experiments in Ventanas (metal polluted) and Quintay (pristine), in central Chile for 10 days. Different letters in bars represent significant differences at the 95% confidence interval ($p < 0.05$). Error bars are ± 1 SD, n = 3.

5.3.4. Antioxidant enzymes

SOD activity was highest in Es147 grown in Ventanas ($p < 0.05$). Although significantly lower activity was found in Es524 than Es147 in Ventanas ($p < 0.05$), activity was significantly higher ($p < 0.05$) than both strains grown in Quintay (Figure 5.8a). The activity of CAT was significantly lower in Es147 that Es524 at both locations and did not differ between Quintay and Ventanas ($p = 0.98$). CAT activity in Es524 was significantly higher ($p < 0.05$) under the conditions encountered in Ventanas (Figure 5.8b). On the other hand, a significant increase in the activity of APx was only apparent in Es524 from Ventanas (Figure 5.8c). Similarly, the only significant ($p < 0.05$) change in GR was the higher activity in Es524 grown in Ventanas (Figure 5.8d).

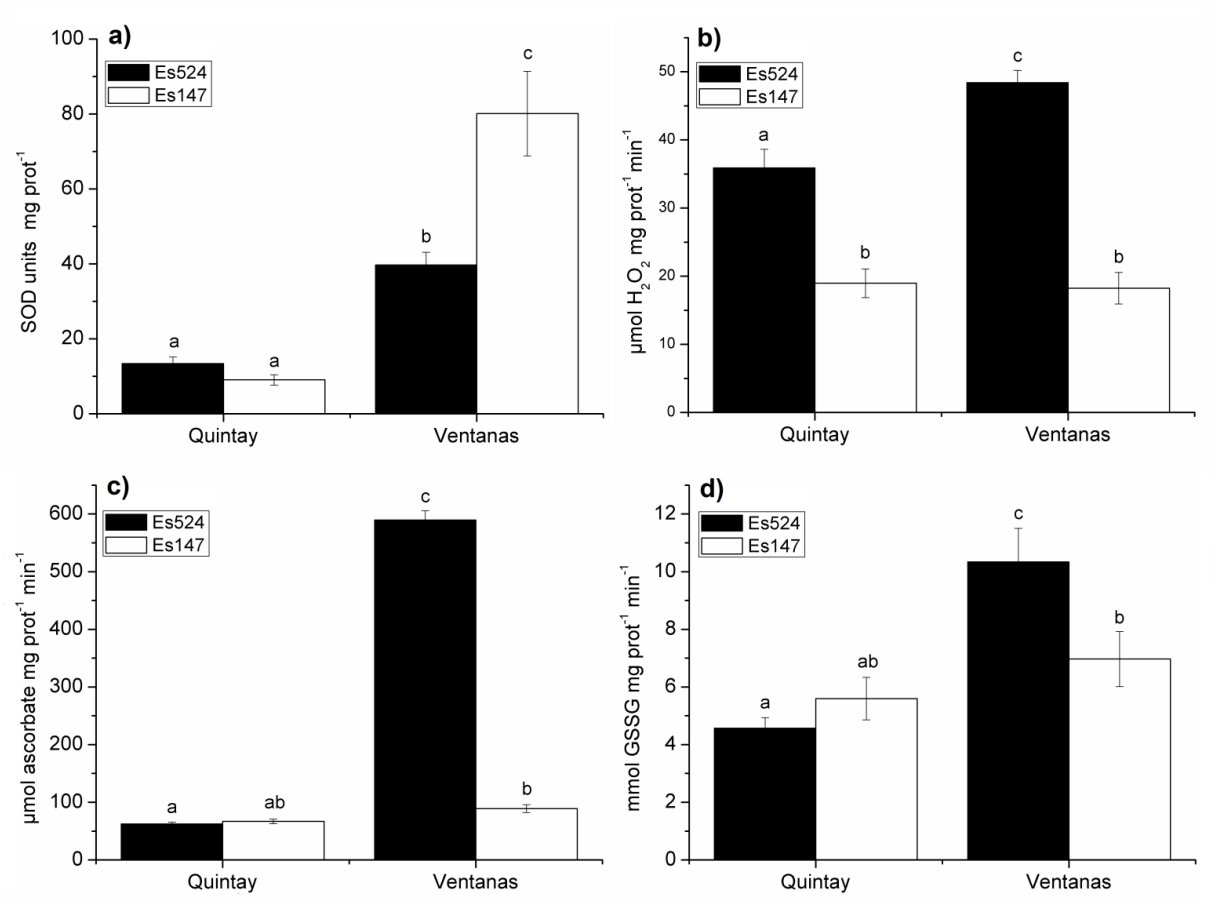

Figure 5.8. Activity of antioxidant enzymes in two *Ectocarpus siliculosus* strains after transplantation experiments in Ventanas (metal polluted) and Quintay (pristine), in central Chile: a) Superoxide dismutase; b) Catalase; c) Ascorbate peroxidase; d) Glutathione reductase. Different letters in bars represent significant differences at the 95% confidence interval ($p < 0.05$). Error bars are ± 1 SD, n = 3.

5.4. Discussion

Our data on metal accumulation was in agreement with a recent metal assessment performed by passive-biomonitoring on local populations of the kelp *Lessonia trabeculata* in Ventanas, which presented higher concentrations of Al, Fe, Pb, and Cu than a pristine location (Sáez et al., 2012a). In contrast, concentrations of Cd were higher in the material grown at Quintay, in agreement with previous findings (Sáez et al., 2012a; Sáez et al., 2012b). Quintay is in close proximity to one of the most important upwelling zones in the South-Western Pacific coast (Aiken et al., 2008). Cd is complexed by nitrates and phosphates in marine waters and then removed by phytoplankton' (Abe and Matsumaga, 1988; Abe, 2001); after sedimentation of the biogenic material and following biological and oceanographic processing, Cd is transported to coastal areas by upwelling (Bruland, 1980). Relating to our results obtained in the laboratory in Chapter III, total Cu accumulation in Es147 and Es524 in Quintay were similar to what we observed in Es524 and LIA4A under 0.4 and 0.8 µM Cu. On the other hand, both strains at Ventanas presented notably higher total Cu accumulation than the strains assessed in Chapter III exposed to 2.4 µM Cu. The information suggests that during the course of the transplantation experiments, the bioavailable fraction of the Cu in the field was much higher than the greatest Cu exposure used in the laboratory experiments; moreover, taking into account that in the field there might have occurred competition between different metals for binding sites extra- and intra-cellularly. We could not observe major patterns in total metal accumulation between strains. It might be that, as observed between Es524 and LIA4A under Cu exposure in the laboratory, the differences are more related to the distribution of metals between adsorbed and intracellular fractions.

The levels of the ROS H_2O_2 increased only for the strain Es147 in Ventanas in relation to Quintay. Instead, Es524 had no changes in the concentrations of H_2O_2, similar to what we observed under Cu exposure in the laboratory. Exposure to elevated concentrations of metals such as Cu can induce increased production of ROS with subsequent increases in lipid peroxidation (LPX), as observed in *Lessonia nigrescens* and *Scytosiphon lomentaria* under 1.6 µM Cu for 4 days (Contreras et al., 2009). Our results in LPX in Es524 in the field are similar to what we observed in this same strain under Cu exposure in the laboratory, with no significant changes under increasing levels of Cu of up to 2.4 µM Cu (Section 3.3.1). On the other hand, Es147 displayed an increase in LPX in Ventanas in relation to Quintay, similar to what we observed in LIA4A under 0.4 and 0.8 µM Cu, and in REP10-11 exposed to 2.4 µM Cu (Section 3.3.1). As confirmed in our field experiments, the strain Es524 appears to have strong antioxidant defences in order to maintain the levels of H_2O_2 and to avoid an increase of LPX under metal stress.

Our results showed that levels of Chl*a* and Chl*c* decreased only for Es147 in Ventanas in relation to Quintay, but not for Es524. A decrease in the levels of Chl might be related to Cu competition with Fe for its synthesis, as observed by Patsikka et al. (2002) in the plant *Phaseolus vulgaris*, or by substitution of Cu by Mg in the Chl, observed in E. siliculosus by Küepper et al. (2002). However, since the same amount of Cu was accumulated by both strains, and no differences were observed in the concentrations of chlorophyll *a* and *c* in Es524 between Quintay and Ventanas, it is more likely that Es147 was less capable of buffering ROS excess than Es524 with a resultant oxidation of Chl. In terms of fucoxanthin, there was an increase in the levels for Es524 in Ventanas in relation to Quintay, but not for Es147. Fucoxanthin is the main xanthophyll pigment in brown algae, and is responsible for their brown colour (Evstigne and Paramono, 1974). As described in the discussion of Chapter IV, fucoxanthin has been described as a strong

antioxidant; however, nothing has been published on its role as antioxidant for brown algae metabolism. In our laboratory experiments with Cu exposure, we observed a systematic increase in the levels of fucoxanthin only in the strain Es524 (Section 3.3.1). Higher levels of fucoxanthin in Es524 in Ventanas follow a similar pattern as observed in our Cu laboratory experiments, suggesting it is acting as an antioxidant. The latter needs to be further tested in the laboratory.

The systematic increase in the concentrations of GSSG in both strains and the decrease in ratios of GSH/GSSG in Ventanas compared to Quintay, provides strong evidence for an oxidative response to the prevailing environmental conditions. Results were similar with those reported for the seaweed *Scytosiphon lomentaria* in Cu polluted locations (Contreras et al., 2005), and also in vascular plants under different environmental stressors (Mhamdi et al., 2010; Noctor et al., 2012). However, higher levels of GSH in Ventanas than in Quintay for both strains suggest that, at these levels of exposure, glutathione recycling is efficient in order to maintain GSH levels stable. A similar pattern could be observed in the strains from metal polluted locations (Es524 and REP10-11) exposed up to 0.8 µM Cu, described in Section 3.3.2.

Regarding ascorbate, there was a clear Asc oxidation to DHA in both strains in Ventanas. These results were similar to the observed in *L. nigrescens* and *S. lomentaria* (Contreras et al., 2005; Contreras et al., 2009), and the green alga *Ulva compressa* (Mishra et al., 1993; Mellado et al., 2012) where levels of DHA also increased under metal stress. This suggests Asc is being oxidized to DHA in order to buffer ROS excess. In addition, the increase in Asc levels in both strains in Ventanas suggest that they can synthesize more Asc to counteract oxidative stress, as it was observed by Ratkevicius et al. (2003) in *U. compressa* in Cu polluted locations. Levels of Asc and DHA in Es524 and Es147 in

Ventanas and even in Quintay were notably higher than in all strains (Es524, REP10-11, and LIA4A) tested in our Cu exposure laboratory experiments; this might be explained by metal-additive or -synergistic effects in the field, or the inclusion of other not identified environmental stressors. Besides the latter, Es524 and Es147 seem to display proficient glutathione-ascorbate interactions, given by good amounts of GSH in Ventanas that might be contributing to efficient reduction of DHA to Asc, in a dehydroascorbate reductase catalysed reaction (DHAR); similar patterns could be observed in *U. compressa* from Cu-polluted areas of central Chile (Ratkevicius et al., 2003).

The behaviour of phenolic compounds was also similar between strains, with higher levels in Ventanas than Quintay; however, total phenolics were the highest in Es147 in Ventanas. In addition to the antioxidant properties of phenolic compounds, these secondary metabolites have been recognized to be good metal chelators extra- and intra-cellularly, and therefore highly important for metal stress metabolism in brown algae (Connan and Stengel, 2011). The pattern of increase in total phenolics in Es524 was similar to our results in with this same strain under Cu exposure of up to 2.4 µM (Section 3.2.2), but in E147 in Ventanas the levels of total phenolics were notably higher than in all the strains tested in the laboratory, even at the highest Cu exposure. The information suggests that production of phenolic compounds increased for both strains in Ventanas to counteract the effects of metal excess and, moreover, might indicate that Es147 is better prepared against metal stress in terms of phenolics syntheses.

Although we observed that both strains increased the activity of SOD in Ventanas, the highest levels of activity were recorded in Es147; this information suggests that more SOD is needed in $\bullet O_2^-$-stressed Es147 in Ventanas, which might lead to a greater production of H_2O_2. The latter is likely to have occurred as manifested in significantly higher levels of

H_2O_2 only in Es147 in Ventanas. Moreover, trends in SOD activity are similar to what we observed in the laboratory experiments, in which the activity increased for all strains tested up to 2.4 µM Cu (Section 3.3.3).

Even though content of Asc increased for both strains in Ventanas in relation to Quintay, suggesting good Asc supply for APx (Yilmaz et al., 2010), its activity was only higher in Ventanas than in Quintay for the strain Es524; in case of CAT the pattern was similar. These results resemble our findings in the laboratory under exposure to up to 2.4 µM Cu, where the strain Es524 only increased both CAT and APx (Section 3.3.3). Both enzymes target H_2O_2, and it has been observed that they cooperate against this ROS in brown algae (Collen and Davison, 2001; Contreras et al., 2009), and also in vascular plants (Asada, 1992; Mizuno et al., 1998). For example, Contreras et al. (2009) found that APx and CAT cooperate in increasing their activities in *L. nigrescens* and *S. lomentaria* under up to 1.6 µM Cu; however, the activities of both CAT and APx were higher in *L. nigrescens* than in *S. lomentaria*, showing that the activities of these enzymes can be highly species-specific. In addition to the records available on inter-species variation in the activities of APx and CAT in different groups of phototrophs, our results in the field (in addition to our laboratory findings) suggest that the activity of both enzymes can be also population-specific, in this case showing that the strain Es524 is more prepared against H_2O_2 stress than Es147. These results were in agreement with the data on H_2O_2 content, where only the strain Es147 presented higher levels of H_2O_2 in Ventanas in relation to Quintay.

The enzyme GR is critical in glutathione recycling by catalysing the reduction of GSSG back to GSH (Noctor et al., 2012). Passive-biomonitoring experiences in other algae such as in *S. lomentaria* (Contreras et al., 2005), and in the *U. compressa* (Mellado et al.,

2012), have found lower levels of GR activity in polluted locations in relation to control areas in northern Chile, maybe showing signs of saturation due to high metal stress. Instead, we observed increased levels of GR activity in the polluted site in relation to Quintay, but only for the strain Es524; this might suggest that either free metals were higher in the locations assessed by the authors described before, or that *E. siliculosus*, and Es524 in particular, is more prepared against metal stress. Differences in the activity of GR between Es524 and Es147, in addition to the other antioxidant enzymes measured in the field and in the laboratory, show that these antioxidant mechanisms can be highly population-specific in brown algae and likely to be inherited after a long history of metal exposure.

There is evidence that populations of some brown seaweeds with different histories of exposure to metal stress can develop resistant ecotypes. For example, Nielsen et al. (2003) observed that different populations of *Fucus serratus* from a metal polluted and pristine locations of South-West England exposed to up to 844 nM Cu^{2+} for 12 and 23 d, revealed intra-specific responses in physiology and metabolism; these authors found that populations with origin in pristine location had lower growth rates in embryos and adults, and higher inhibition of photosynthesis, compared to 'resistant' populations. More recently in a proteomic study, Ritter et al. (2010) performed Cu exposure experiments on two strains of *E. siliculosus*, Es524 (also included in our investigation) and Es32 (from a pristine location in Peru). They observed that the Mn-stabilizing protein of the oxygen evolving complex (OEC33) of PSII was more expressed in Es524 than in Es32 exposed to 0.8 µM Cu for 10 d. They suggested that this overexpression can result in better recycling of OEC33 damaged by ROS excess in Es524. Finally, our findings described in Chapters III and IV in *E. siliculosus* under Cu excess of up to 2.4 µM Cu also support the intra-specific inherited metal resistance, as manifested in better Cu exclusion mechanisms,

higher production and polymerization of phytochelatins, and a strong ROM in the strain Es524 (with origin in a Cu polluted location. The latter information on inter-population variability in metal resistance and response needs to be considered when conducting biomonitoring using brown algae, because differences in stress response between populations, and non-informed decisions in appropriate biomarkers, can lead to false environmental diagnosis. Our investigation provides the first description of inter-population differences in metal-mediated oxidative stress responses through field experiments in brown algae, and showed that Es524 is more metal tolerant than the strain Es147. Despite the inherent differences in metal response observed in different strains of *E. siliculosus* under laboratory and field experiments, they seem to display similar patterns in some of the parameters we have tested in relation to their ROM; thus, these might represent good inter-population biomarkers that can successfully represent metal environmental status.

Metal accumulation was similar between strains and displayed levels of accumulation in agreement with the records of metal contamination in Ventanas (Sáez et al., 2012a; Sáez et al., 2012b). Levels of H_2O_2 and LPX were higher in Ventanas than in Quintay in Es147, but not for Es524. Chlorophyll *a* and *c* content decreased in Es147 in Ventanas in relation to Quintay, but not in Es524. Finally, fucoxanthin concentrations are higher in Es524 in Ventanas, but not in Es147. On the other hand, an increase in the levels of glutathione and ascorbate, their interactions, and relations between reduced and oxidised forms, showed clear signs of environmental stress in Ventanas in relation to Quintay, in both strains. Higher levels of phenolic compounds in Ventanas in both strains were also evidence of environmental stress. In terms of the antioxidant enzymes measured, the activity of all of them was higher in Es524 in Ventanas than in Quintay, which in case of the strain Es147 was only observed for the enzyme SOD. The Table 5.5 shows a summary

of resistance between strains, similar to Table 3.2 in Chapter IV. The transplantation method designed was successful in order to represent metal exposure and dose in the locations assessed and, in agreement with the available literature (Neaman et al., 2009; Sáez et al., 2012a; Sáez et al., 2012b), showed that Ventanas was metal polluted. However, in order to reduce errors induced by inter-population differences in response, only those biomarkers that displayed similar behaviour between strains, and represented clear signs of environmental stress, are the ones that should be considered for future assessments. The information suggests that the best biomarker candidates for future environmental evaluations with *E. siliculosus* are: metal accumulation, glutathione and ascorbate in reduced and oxidized forms, content of phenolic compounds, and the activity of SOD.

Table 5.5. Summary of Cu resistance related parameters in the two *Ectocarpus silicuosus* strains assessed after transplantation experiments for 10 d in a metal-polluted (Ventanas) and pristine (Qunitay) locations of Central Chile; also see Table 3.2 in Chapter IV. Es524 is originally from a location with history of Cu pollution in Chile; the strain Es524 is from northern Chile, an area with history of recent low Cu pollution. More + symbols indicate that the strain was better able to cope with Cu-induced oxidative stress based on the respective parameters investigated. For example, strain Es524 showed low levels of lipid peroxidation, but high levels of ascorbate peroxidase and glutathione reductase. In conclusion, Cu resistance was ranked as: Es524 > Es147.

Oxidative stress indicators	**Es524**	**Es147**
H_2O_2 levels	+++	+
Lipid peroxidation	+++	+
Oxidation of pigments	+++	+
Antioxidants		
Glutathione	++	++
Ascorbate	++	++
Phenolic compounds	++	+++
Fucoxanthin as antioxidant	+++	+
Activity of antioxidant enzymes		
Superoxide dismutase	++	+++
Ascorbate peroxidase	+++	+
Catalase	+++	+
Glutathione reductase	+++	+

Our results suggest that this protocol can be applied in ecosystems with low to high metal pollution and, moreover, in locations lack *E. siliculosus* and a hard/rocky substrate. Furthermore, the worldwide distribution of *E. siliculosus*, its easy cultivability and fast growth, the short periods of exposure of the device in the field, and the low cost of the transplantation device and analytical and biochemical analyses, highlight this novel protocol as a simple, cost-effective method, that provides a reliable representation of metal environmental status.

Chapter VI

A simple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass *Ectocarpus siliculosus*, the model brown alga

6.1. Introduction

The brown algae are an ecologically and economically important group of marine photoautotrophs (Smith, 1996b; Smith, 1996a; Villegas et al., 2008; Sáez et al., 2012b) that first appeared some 200 million years ago and evolved multicellularity independently of green and red algae and higher plants. In 2010, the genome of *Ectocarpus siliculosus* (Dillwyn) Lyngbye, a filamentous brown alga of the order Ectocarpales, was published (Cock et al., 2010) and it has been proposed as a model organism for brown algal genetic and genomic studies (Peters et al., 2004a). The species has certain characteristics such as a relative small genome of 214 Mbp (Cock et al., 2010), a short life cycle that can be completed in laboratory culture (Müller et al., 1998), fast growth and ease of performing genetic crosses (Peters et al., 2004a; Peters et al., 2008), that makes it amenable to emerging molecular technologies.

So far, over 1500 strains of *E. siliculosus* have been isolated, from a broad range of geographical locations and ecological niches, and are maintained in culture collections (Gachon et al., 2007; Dittami, S. M. et al., 2011; Gachon et al., 2013). They, therefore, provide a valuable resource for investigation the molecular mechanisms underlying the dynamic responses of brown algae to abiotic and biotic stressors.

To perform molecular characterization, a wide range of approaches are available (e.g. RT-PCR, qRT-PCR, microarray, cDNA library construction, SNP genotyping, DNA methylation profiling and next-generation sequencing), all requiring DNA and RNA samples of high purity (Wink, 2006). The extracted nucleic acids need to be free of contaminants, including proteins, carbohydrates and lipids, but also of other nucleic acids; for example, it is important to obtain DNA free of RNA or RNA free of DNA, respectively

(Buckingham and Flaws, 2007). Quality and integrity of the isolated nucleic acids will directly affect the results of all dowstream applications (Cseke et al., 2004).

Special precautions are required for RNA isolation as it has a very short half-life once extracted from cells or tissues and is susceptible to degradation (Kojima and Ozawa, 2002; Buckingham and Flaws, 2007). RNA is especially unstable due to the ubiquitous presence of RNase enzymes which can be delivered in the environment by different organisms, from man to bacteria and fungi (Brooks, 1998; Buckingham and Flaws, 2007). Strong denaturants, suitable to inhibit endogenous RNases, are used to isolate intact RNA (Doyle, 1996). The extracted RNA includes all naturally occurring RNA present in cells: ribosomal RNA (rRNA) (80%–90%), messenger RNA (mRNA) (2.5%–5%) and transfer RNA (tRNA) (Buckingham and Flaws, 2007). Therefore, to perform downstream studies related to transcriptomic profiles, mRNA must be further purified from the pool of RNA through polyA selection.

As for genomic DNA, high quality DNA is a pre-requisite for performing downstream applications such as high throughput sequencing, which allow scientists to generate large quantities of sequence data in a short period of time. Each step of this analysis is exacerbated by degraded DNA that can result in loss of regions of the genome.

Currently, there are many specialized solution-based or column-based protocols for the extraction of pure DNA and RNA. Most of these protocols have been developed into commercial kits that ease the extraction procedures. Although guanidine isothiocyanate-based extraction buffers (e.g. TRIzol® reagent, Invitrogen, Carlsbad, CA, USA) and several commercial kits (RNeasy® kit, Qiagen,Valencia, CA, USA), are commonly used for high quality nucleic acid extraction in model plants, they are

unsuitable for organisms containing high levels of starch, polysaccharides and polyphenols (Chomczynski and Sacchi, 1987). Polysaccharides can co-absorb nucleic acids thus resulting in reduced yields and poor quality extracts, which, in the case of DNA, will interfere with endonuclease digestion (Wilkins and Smart, 1996; Li and Trick, 2005; Wang et al., 2005; Hoarau et al., 2007). Also, high concentrations of phenols, which can be co-extracted with nucleic acids and constitute strong enzyme inhibitors, can significantly impact the extraction procedure (Mayes et al., 1992; Jin et al., 1997). Thus, it is not surprising that for algae, and in particular brown algae, which are particularly rich in problematic biomolecules such as lipids, polysaccharides (e.g. cellulose, alginates and sulfated fucans), and polyphenolic compounds, the isolation of pure nucleic acids represents a major challenge (Michel et al., 2010).

At present, several protocols are available for extracting RNA from brown algae, including one for a specific strain of *E. siliculosus* (unialgal strain 32, CCAP accession 1310/4, origin San Juna de Marcona, Peru) (Pearson et al., 2006; Le Bail et al., 2008b; Dittami et al., 2009; Yao et al., 2009). However, because the concentrations of problematic biomolecules can vary between different strains and genotypes of E. *siliculosus,* the success of nucleic acid isolation is likely to be strain-/genotype-dependent. Therefore, modified protocols may need to be developed in order to deal with material containing different quantities of metabolites. An additional problem is obtaining sufficient amounts of biomass for performing biochemical and molecular analyses. *E. siliculosus* is a small filamentous alga that grows to a length of about 30 cm and does not produce much biological material during short-term experimental studies (Peters et al., 2008). Existing protocols for obtaining good yields of DNA from *E. siliculosus* require 1g of biomass (Coelho et al., 2012a). Therefore, developing a protocol that uses less biomass for nucleic

acid extraction or the co-isolation of DNA and RNA from the same material would represent a significant breakthrough.

Thus, to address the issues of the purity of extracted nucleic acid, availability of small quantities of biomass and strain-wide efficiency a rapid and effective method for the co-extraction of high-quality DNA and RNA that is applicable for different strains of *E. siliculosus* of low biomass (25-, 50- and 100mg) has been developed. Moreover, in order to compare this new protocol with one previously used for *E. siliculosus* based on CTAB extraction buffer (Le Bail et al., 2008b), samples were processed according to both methods.

6.2. Materials and Methods

6.2.1 Overview of the technique

This procedure employs a high pH extraction buffer (pH 9.5) which contains 100 mM Tris-HCl, plus the addition of 5 mM DTT and 1% sarkosyl, which effectively inhibits RNase activity during the extraction, and removes most polysaccharides, polyphenols and other insoluble material. Sodium acetate was used along with isopropanol for precipitation to enhance the DNA/RNA yields and shorten the precipitation time, as shown in Figure 6.1. A phenol:chlorophorm: isoamyl alcohol step was subsequently used to purify the nucleic acids. To simplify the understanding of the methodology and results, details of the consumables, reagents, equipment, and the recipe for the extraction and purification of nucleic acids, was included in the Appendix 2 of this Thesis.

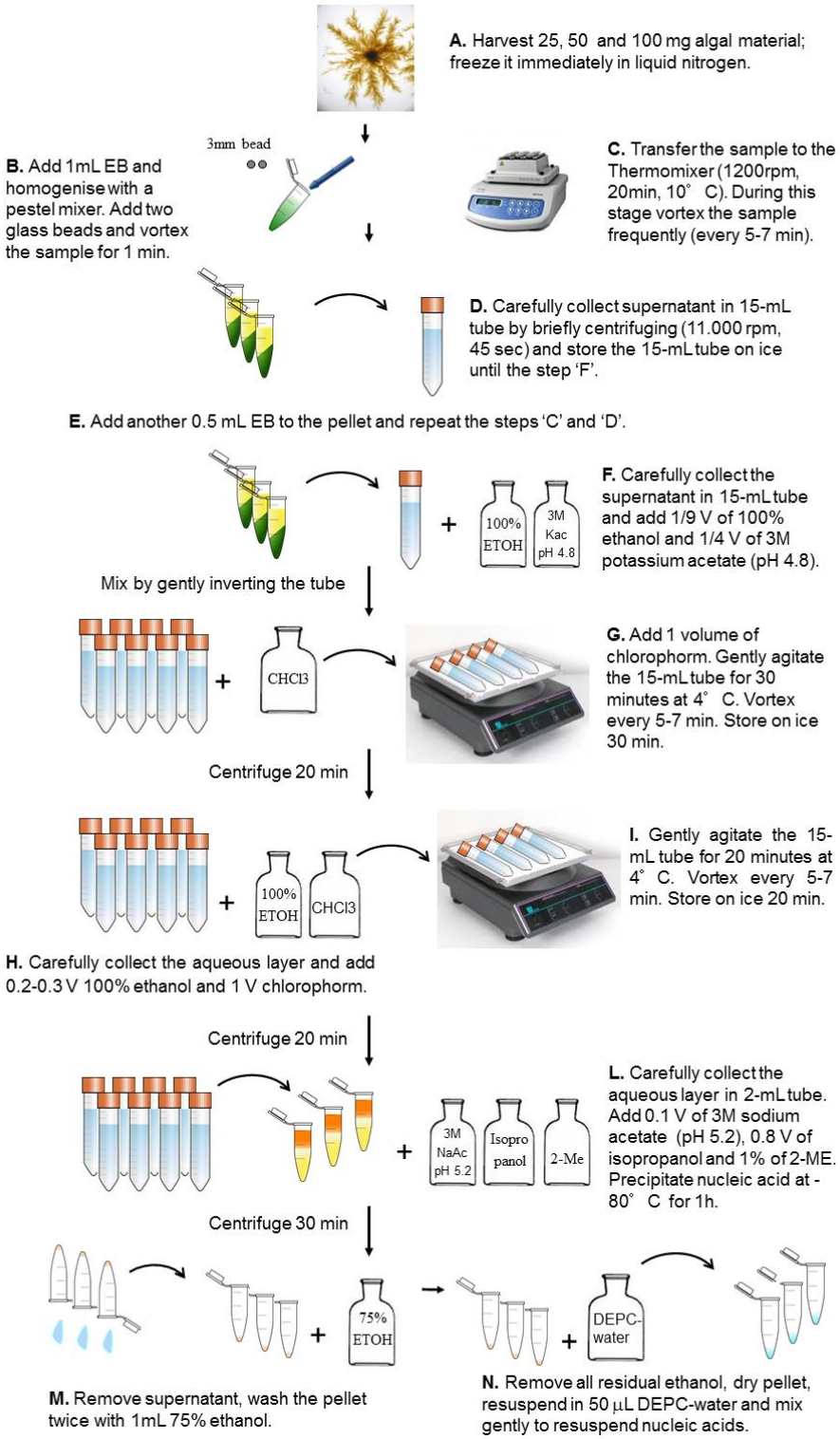

Figure 6.1. Summary of the extraction protocol for nucleic acids from *E. siliculosus.*

6.2.2. Measuring DNA/RNA concentration and quality

Total DNA/RNA solutions, extracted from 25-100 mg of algae, were loaded on a 1.5% agarose gel for electrophoresis, stained with ethidium bromide (EtBr), and visualized under UV light to assess the quality and integrity of nucleic acids. Nucleic acid quantification was carried out placing 1.5-2 μL in a Nanodrop spectrophotometer, which provided the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230}, which can be used to assess presence of protein and polysaccharide/polyphenolic contamination (Logemann et al., 1987; Manning, 1991; Asif et al., 2000; Manickavelu et al., 2007).

NOTE: DNA/RNA concentration and purity can also be determined spectrophotometrically by measuring the absorbance at 230, 260 and 280 nm (Sambrook and Russel, 2001).

6.2.3. Nucleic acids downstream applications

a) Reverse transcription

Total RNA (1 μg) from each sample was reverse transcribed with the SuperScript III reverse transcriptase and oligo dT(22) according to the manufacturer's instructions (Invitrogen, Milan).

b) DNA and cDNA amplification

To test DNA/cDNA quality, amplification was performed according to the following protocol (Table 6.1). For multiple samples, a master mix can be prepared.

Table 6.1. PCR mix components used in DNA/cDNA amplification

Component	**Volume**
5X Taq DNA polymerase buffer with $MgCl_2$	10 µL
10mM dNTP mix	1 µL
10µM TUA.FW primer	1.5 µL
10µM TUA.BW primer	1.5 µL
10U/µl GoTaq DNA polymerase (Promega)	0.25 µL
70-100 ng template DNA/cDNA	1 µL
Water	Up to 50 µL

DNA/cDNA amplification was performed using Alpha Tubulin (TUA) housekeeping gene (Le Bail et al., 2008b); primer sequences are reported in Table 6.2. PCR reactions were performed according the cycle profile described in Table 6.3.

Table 6.2. Alpha Tubulin (TUA) primers sequences

Primers	**Sequence (5'-3')**
TUA.FW	TTTGAGGAGTTTCGTCGGAGAT
TUA.BW	CACACAGCGCAAAACGGC

Table 6.3. Run PCR program

Cycling Conditions	
Start	95 °C, 2 min
Step 1-35	95°C, 50 s
	54°C, 50 s
	72°C, 50 s
Final extension step	72°C, 7 min
Hold	4°C

The PCR products (25 µL) were resolved on a 1% agarose gel, prepared in TBE 0.5% buffer, and visualized under UV light following EtBr staining.

6.2.4. Nucleic acids downstream applications: DNA digestion

DNA samples were digested according the protocol applied for Southern blot DNA digestion (Table 6.4). For multiple samples, a master mix can be prepared.

Table 6.4. Restriction enzyme digestion of DNA

Component	**Amount**
Genomic DNA	5-10 µg
10U/µl EcoRV enzyme (Fermentas, Italy)	60 U
10X EcoRV enzyme buffer	20 µL
Optionally: BSA (10µg/µL)	2 µL
Water	Up to 200 µL

Samples were incubated overnight at 37°C. The reaction was stopped by incubating at 65°C for 10 minutes. The digested DNA was precipitated at –20°C overnight in the presence of 0.1V of 3M sodium acetate (pH 5.2) and 2.5 V cold 100% ethanol. Samples were centrifuged at 13,000 rpm for 20 min at 4ºC. The DNA pellet was washed with 1mL of cold 75% ethanol, dried and re-suspended in 50µl water. A 20 µL aliquot was rapidly checked by electrophoresis.

6.3. Results

6.3.1. Yield of genomic DNA and total RNA

As a general rule, after the purification step, the total amount (µg) of nucleic acids decreased by approximately 40-50%, with a simultaneous increase in purity, regardless of the amount of biomass used (observe Tables 6.5, 6.6, and 6.7). However, a higher yield of both DNA and RNA was obtained using the new protocol than the CTAB extraction buffer method (Le Bail et al., 2008b) (Tables 6.6, 6.8). Indeed, a differential decrease in the quantity of nucleic acids was recorded for all strains when the older method was used: on average, 65% (DNA) and 64% (RNA) for REP10-11; 43% (DNA) and 41.6% (RNA) for Es524; 40% (DNA) and 32% (RNA) for RHO12; and 16% (DNA) and 9.6% (RNA) for LIA4A (see Table 6.6, 6.8).

Table 6.5. Comparisons of genomic DNA (A) and total RNA (B) yield and purity obtained from four strains of *Ectocarpus siliculosus,* extracted according the new procedures. Data referred to DNA and RNA yield and purity obtained at the end of step 30. DNA/RNA purities were calculated through the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} used to assess presence of protein and polysaccharide/polyphenolic compound. Total amounts of nucleic acids (µg) were calculated in a final volume of 50 µL (a).

(A) Strain	Average Yield of Fresh Tissue (mg)	$A_{260/280}$	$A_{260/230}$	DNA conc. (ng/µl)	Total DNA (µg)[(a)]	DNA Yield (µg/mg)
REP 10.11	25	1.82 ± 0.05	1.93 ± 0.05	198.8 ± 3.75	9.94 ± 0.78	0.397 ± 0.025
	50	1.82 ± 0.04	1.82 ± 0.08	244.8 ± 4.62	12.24 ± 0.84	0.245 ± 0.018
	100	1.81 ± 0.03	1.72 ± 0.04	489.2 ± 6.92	24.46 ± 0.72	0.245 ± 0.026
Es524	25	1.84 ± 0.06	1.61 ± 0.06	169.4 ± 5.72	8.47 ± 0.50	0.339 ± 0.035
	50	1.73 ± 0.04	1.44 ± 0.03	278.9 ± 4.68	13.94 ± 0.86	0.279 ± 0.023
	100	1.71 ± 0.03	1.34 ± 0.02	404.4 ± 6.73	20.22 ± 0.66	0.202 ± 0.018
LIA4A	25	1.79 ± 0.08	1.57 ± 0.04	366.6 ± 5.88	18.33 ± 0.74	0.733 ± 0.065
	50	1.72 ± 0.04	1.48 ± 0.06	477.9 ± 6.84	23.89 ± 0.94	0.478 ± 0.049
	100	1.70 ± 0.05	1.42 ± 0.08	947.9 ± 5.53	47.39 ± 0.86	0.474 ± 0.044
RHO12	25	1.71 ± 0.08	1.43 ± 0.04	286.2 ± 4.33	14.31 ± 0.46	0.572 ± 0.026
	50	1.70 ± 0.03	1.35 ± 0.06	436.5 ± 9.32	21.82 ± 0.58	0.436 ± 0.045
	100	1.69 ± 0.04	1.35 ± 0.08	556.0 ± 9.82	27.80 ± 0.94	0.278 ± 0.037

(B) Strain	Average Yield of Fresh Tissue (mg)	$A_{260/280}$	$A_{260/230}$	RNA conc. (ng/µl)	Total RNA (µg)[(a)]	RNA Yield (µg/mg)
REP 10.11	25	1.82 ± 0.04	1.92 ± 0.03	174.0 ± 2.73	8.70 ± 0.58	0.348 ± 0.012
	50	1.80 ± 0.08	1.82 ± 0.02	252.8 ± 9.92	12.64 ± 0.67	0.253 ± 0.023
	100	1.76 ± 0.02	1.72 ± 0.05	435.2 ± 8.82	21.76 ± 0.78	0.217 ± 0.015
Es524	25	1.82 ± 0.03	1.53 ± 0.04	113.2 ± 5.82	5.66 ± 0.28	0.226 ± 0.026
	50	1.72 ± 0.05	1.31 ± 0.06	243.7 ± 5.63	12.18 ± 0.26	0.244 ± 0.015
	100	1.68 ± 0.04	1.22 ± 0.06	354.8 ± 6.43	17.74 ± 0.54	0.177 ± 0.034
LIA4A	25	1.75 ± 0.05	1.52 ± 0.04	284.6 ± 4.85	14.23 ± 0.85	0.569 ± 0.047
	50	1.75 ± 0.06	1.46 ± 0.06	374.8 ± 4.92	18.74 ± 0.64	0.375 ± 0.034
	100	1.72 ± 0.08	1.27 ± 0.08	805.4 ± 8.86	40.27 ± 0.72	0.403 ± 0.042
RHO12	25	1.71 ± 0.09	1.45 ± 0.04	202.7 ± 5.82	10.13 ± 0.56	0.405 ± 0.029
	50	1.72 ± 0.05	1.40 ± 0.02	289.3 ± 6.82	14.46 ± 0.47	0.289 ± 0.016
	100	1.72 ± 0.04	1.44 ± 0.05	420.4 ± 7.66	21.02 ± 0.95	0.210 ± 0.024

Table 6.6. Comparison of DNA yield and purity, obtained from four strains of *Ectocarpus siliculosus* by two different methods: the new and old (Le Bail et al., 2008b). Total amounts of nucleic acids were calculated in a final volume of 40 µL (a). Data are reported as means ± SD from more than five independent nucleic acid extractions, for both methods. 'New' refers to the new method developed in this chapter; 'Old' refers to a previously published protocol based on CTAB extraction buffer (Le Bail et al., 2008b).

Strain		Average Yield of Fresh Tissue (mg)	$A_{260/280}$		$A_{260/230}$		DNA conc. (ng/µL)		Total DNA (µg)[(a)]		DNA Yield (µg/mg)	
			New	Old	New	Old	New	Old	New	Old	New	Old
Polluted	REP 10.11	25	2.01 ± 0.02	1.66 ± 0.01	2.20 ± 0.04	1.37 ± 0.01	132.9 ± 4.73	45.0 ± 2.53	5.32 ± 0.38	1.8 ± 0.18	0.213 ± 0.014	0.072 ± 0.006
		50	1.91 ± 0.03	1.54 ± 0.04	2.00 ± 0.04	1.17 ± 0.02	181.4 ± 5.32	69.3 ± 2.76	7.25 ± 0.32	2.9 ± 0.27	0.145 ± 0.017	0.058 ± 0.009
		100	1.86 ± 0.03	1.59 ± 0.03	1.86 ± 0.03	1.15 ± 0.01	389.6 ± 4.52	123.3 ± 4.34	15.58 ± 0.86	4.93 ± 0.48	0.156 ± 0.014	0.049 ± 0.006
	Es524	25	1.96 ± 0.04	1.59 ± 0.01	1.75 ± 0.02	1.36 ± 0.02	96.5 ± 3.82	58.0 ± 2.51	3.86 ± 0.42	2.32 ± 0.45	0.154 ± 0.015	0.093 ± 0.013
		50	1.92 ± 0.02	1.60 ± 0.02	1.66 ± 0.03	1.27 ± 0.02	213.4 ± 6.87	124.3 ± 5.43	8.54 ± 0.55	4.97 ± 0.84	0.171 ± 0.017	0.099 ± 0.014
		100	1.85 ± 0.02	1.56 ± 0.02	1.65 ± 0.03	1.15 ± 0.02	314.6 ± 7.83	139.5 ± 4.82	12.58 ± 0.75	5.58 ± 0.62	0.126 ± 0.016	0.056 ± 0.006
Pristine	LIA4A	25	1.91 ± 0.04	1.25 ± 0.02	1.76 ± 0.03	1.61 ± 0.01	274.7 ± 4.39	226.2 ± 6.92	10.98 ± 0.42	9.05 ± 0.58	0.439 ± 0.029	0.362 ± 0.009
		50	1.87 ± 0.02	1.19 ± 0.01	1.73 ± 0.02	1.62 ± 0.02	357.1 ± 5.64	332.0 ± 7.43	14.28 ± 0.96	13.28 ± 1.34	0.285 ± 0.024	0.265 ± 0.007
		100	1.81 ± 0.04	1.20 ± 0.02	1.73 ± 0.02	1.59 ± 0.02	653.9 ± 6.43	515.4 ± 5.73	26.15 ± 1.28	20.6 ± 2.65	0.261 ± 0.031	0.206 ± 0.008
	RHO12	25	1.83 ± 0.03	1.25 ± 0.03	1.63 ± 0.02	0.69 ± 0.02	207.6 ± 5.62	93.5 ± 3.23	8.30 ± 0.65	3.74 ± 0.83	0.332 ± 0.043	0.149 ± 0.009
		50	1.80 ± 0.03	1.19 ± 0.02	1.60 ± 0.03	0.67 ± 0.02	307.2 ± 4.72	157.9 ± 2.11	12.29 ± 1.04	6.31 ± 0.95	0.246 ± 0.021	0.126 ± 0.01
		100	1.80 ± 0.02	1.15 ± 0.03	1.61 ± 0.03	0.63 ± 0.01	390.6 ± 8.21	253.0 ± 2.43	15.62 ± 1.43	10.12 ± 2.41	0.156 ± 0.043	0.102 ± 0.01

Table 6.7. Comparisons between mean values of pure DNA yield and purity between strains isolated from polluted sites (REP10-11, Es524) and those from pristine sites (LIA4A, RHO12). Strains collected from polluted sites exhibit higher levels of nucleic acid purity as evidenced by the A260/280 and A260/230 ratios, compared to strains from pristine ones; strains collected from pristine sites exhibit higher quantities of nucleic acids extracted compared with those from polluted sites. Total amounts of nucleic acids (µg) were calculated in a final volume of 40 µL (a). Data are reported as means ± SD from more than five independent nucleic acid extractions. 'New' refers to the new method developed in this chapter.

Strain	Average Yield of Fresh Tissue (mg)	$A_{260/280}$	$A_{260/230}$	DNA conc. (ng/µL)	Total DNA (µg) [(a)]	DNA Yield (µg/mg)
		New	New	New	New	New
Mean Value Polluted Sites	25	1.98 ± 0.035	1.97 ± 0.318	114.7 ± 25.7	4.59 ± 1.03	0.183 ± 0.041
	50	1.91 ± 0.007	1.83 ± 0.240	197.4 ± 22.6	7.89 ± 0.91	0.158 ± 0.018
	100	1.89 ± 0.042	1.75 ± 0.148	352.1 ± 53.0	14.08 ± 2.12	0.141 ± 0.021
Mean Value Pristine Sites	25	1.87 ± 0.056	1.69 ± 0.091	241.1 ± 47.4	9.64 ± 1.89	0.385 ± 0.075
	50	1.83 ± 0.049	1.66 ± 0.091	332.1 ± 35.3	13.28 ± 1.40	0.265 ± 0.027
	100	1.80 ± 0.007	1.67 ± 0.084	522.2 ± 186.2	20.88 ± 7.44	0.208 ± 0.074

Table 6.8. Comparison of pure RNA yield and purity, obtained from four strains of *Ectocarpus siliculosus* by two different methods: the new and old (Le Bail et al., 2008b). Total amounts of nucleic acids (µg) were calculated in a final volume of 40 µL (a). Data are reported as means ± SD from more than five independent nucleic acid extractions for both methods. 'New' refers to the new method developed in this chapter; 'Old' refers to a previously published protocol based on CTAB extraction buffer.

Strain		Average Yield of Fresh Tissue (mg)	$A_{260/280}$		$A_{260/230}$		RNA conc. (ng/µl)		Total RNA (µg) [(a)]		RNA Yield (µg/mg)	
			New	Old	New	Old	New	Old	New	Old	New	Old
Polluted	REP 10.11	25	2.01 ± 0.03	1.68 ± 0.01	2.44 ± 0.04	1.36 ± 0.02	110.9 ± 5.14	33.2 ± 2.43	4.44 ± 0.15	1.33 ± 0.09	0.177 ± 0.023	0.053 ± 0.008
		50	1.90 ± 0.02	1.51 ± 0.04	2.00 ± 0.02	1.18 ± 0.04	153.6 ± 7.12	60.3 ± 2.54	6.14 ± 0.34	2.41 ± 0.14	0.123 ± 0.035	0.048 ± 0.005
		100	1.84 ± 0.05	1.64 ± 0.04	1.85 ± 0.03	1.11 ± 0.04	295.4 ± 6.32	113.3 ± 2.16	11.82 ± 0.32	4.53 ± 0.23	0.118 ± 0.024	0.045 ± 0.006
	Es524	25	1.96 ± 0.03	1.64 ± 0.02	1.75 ± 0.04	1.34 ± 0.06	66.5 ± 3.45	44.2 ± 3.12	2.66 ± 0.13	1.77 ± 0.12	0.106 ± 0.012	0.07 ± 0.007
		50	1.90 ± 0.04	1.62 ± 0.06	1.63 ± 0.06	1.25 ± 0.03	184.8 ± 5.14	110.3 ± 2.54	7.39 ± 0.45	4.41 ± 0.23	0.148 ± 0.024	0.088 ± 0.009
		100	1.84 ± 0.04	1.52 ± 0.05	1.62 ± 0.02	1.12 ± 0.02	271.0 ± 7.30	129.0 ± 3.23	10.84 ± 0.45	5.16 ± 0.25	0.108 ± 0.014	0.052 ± 0.007
Pristine	LIA4A	25	1.89 ± 0.06	1.25 ± 0.04	1.73 ± 0.02	1.62 ± 0.03	155.6 ± 7.02	124.2 ± 4.32	6.22 ± 0.37	4.97 ± 0.23	0.249 ± 0.051	0.2 ± 0.04
		50	1.87 ± 0.07	1.16 ± 0.05	1.71 ± 0.02	1.60 ± 0.02	233.5 ± 6.25	227.0 ± 6.01	9.34 ± 0.43	9.08 ± 0.34	0.187 ± 0.034	0.18 ± 0.03
		100	1.79 ± 0.01	1.20 ± 0.03	1.71 ± 0.06	1.58 ± 0.04	514.7 ± 10.2	475.4 ± 4.53	20.5 ± 0.33	19.0 ± 0.43	0.205 ± 0.023	0.19 ± 0.04
	RHO1 2	25	1.84 ± 0.04	1.24 ± 0.04	1.63 ± 0.04	1.08 ± 0.01	154.2 ± 6.12	88.3 ± 3.12	6.17 ± 0.34	3.52 ± 0.13	0.247 ± 0.024	0.14 ± 0.03
		50	1.83 ± 0.02	1.17 ± 0.03	1.60 ± 0.06	1.09 ± 0.02	174.7 ± 5.17	131.0 ± 5.43	6.98 ± 0.30	5.24 ± 0.21	0.139 ± 0.022	0.10 ± 0.02
		100	1.86 ± 0.06	1.16 ± 0.06	1.62 ± 0.04	1.06 ± 0.02	311.6 ± 7.43	249.0 ± 7.32	12.46 ± 0.34	9.96 ± 3.23	0.125 ± 0.012	0.10 ± 0.02

Table 6.9. Comparisons between mean values of RNA yield and purity between strains isolated from polluted sites (REP 10.11, Es524) and those from pristine sites (LIA4A, RHO12). Strains collected from polluted sites exhibit higher levels of nucleic acid purity as evidenced by the $A_{260/280}$ and $A_{260/230}$ ratios, compared to strains from pristine ones; strains collected from pristine sites exhibit higher quantities of nucleic acids extracted compared with those from polluted sites. Total amounts of nucleic acids (µg) were calculated in a final volume of 40 µL (a). Data are reported as means ± SD from more than five independent nucleic acid extractions. 'New' refers to the new method developed in this chapter.

Strain	Average Yield of Fresh Tissue (mg)	$A_{260/280}$	$A_{260/230}$	RNA conc. (ng/µL)	Total RNA (µg) [(a)]	RNA Yield (µg/mg)
		New	New	New	New	New
Mean Value Polluted Sites	25	1.98 ± 0.03	2.09 ± 0.48	88.7 ± 31.4	3.55 ± 1.25	0.141 ± 0.05
	50	1.90 ± 0.00	1.81 ± 0.26	169.2 ± 22.0	6.76 ± 0.88	0.135 ± 0.01
	100	1.84 ± 0.00	1.73 ± 0.16	283.2 ± 17.2	11.33 ± 0.69	0.113 ± 0.007
Mean Value Pristine Sites	25	1.86 ± 0.48	1.68 ± 0.07	154.9 ± 0.98	6.19 ± 0.03	0.248 ± 0.001
	50	1.85 ± 0.02	1.65 ± 0.05	204.1 ± 41.5	8.16 ± 1.66	0.163 ± 0.033
	100	1.82 ± 0.03	1.66 ± 0.04	413.1 ± 143.6	16.48 ± 5.68	0.165 ± 0.056

With the newly developed protocol, nucleic acid yields varied with initial quantity of biomass and between strains (Table 6.6, 6.7). For all strains, the absolute amount (μg) of purified nucleic acids extracted from 100 mg biomass was higher than that from 50- and 25-mg biomass. However, when quantities of nucleic acids were normalized to biomass (i.e. μg mg^{-1} of fresh weight) yield (μg mg^{-1}) was higher from 25- than 50- and 100-mg biomass for three of the strains (RHO12; LIA4A; REP10-11) and from 50 mg for Es524. These results are consistent with a complete disintegration of tissue/cell structure in the extraction buffer when low biomass (e.g. 25- and 50-mg) is used compared with the largest biomass (100 mg).

Strain-dependent differences in final yields were also observed. The mean yields of nucleic acids (μg mg^{-1}), extracted from equal initial biomass, were higher in strains collected from pristine sites (LIA4A > RHO12) than from polluted sites (REP10-11 > Es524) (Table 6.6, 6.7, 6.8, 6.9).

6.3.2. Purity of genomic DNA and total RNA

For all strains analyzed, the A_{260}/A_{280} and A_{260}/A_{230} ratios improved following the RNase/DNase treatment and purification steps (Tables 6.5, 6.6, 6.7, 6.8, 6.9). Furthermore, the quality of nucleic acids obtained by this method was significantly better than that described by Le Bail et al. (2008b).

The purity of nucleic acids depended on both the quantity of initial biomass used and the strain. In general, 25mg biomass provided the highest level of purity and when used in the co-isolation, the A_{260}/A_{280} ratios ranged between 1.8 and 2.0, whilst the A_{260}/A_{230} ratios ranged between 1.6 and 2.4 (Tables 6.6, 6.7, 6.8, 6.9), indicating that the

DNA and RNA samples were effectively separated from both proteins and polysaccharides (Tables 6.6, 6.7, 6.8, 6.9). A comparison of the mean values of the A_{260}/A_{280} and A_{260}/A_{230} ratios indicates that the two strains collected from polluted sites (REP10-11, Es524) had higher levels of purity than strains from the pristine sites (LIA4A, RHO12). For REP10-11, the A_{260}/A_{280} and A_{260}/A_{230} ratios ranged between 1.8 and 2.0, and 1.8 and 2.4, respectively, and independent of the quantity of biomass used (Figure 6.2). Regardless of the amount of biomass or strain used, the nucleic acids extracted through this protocol were successfully used for downstream applications.

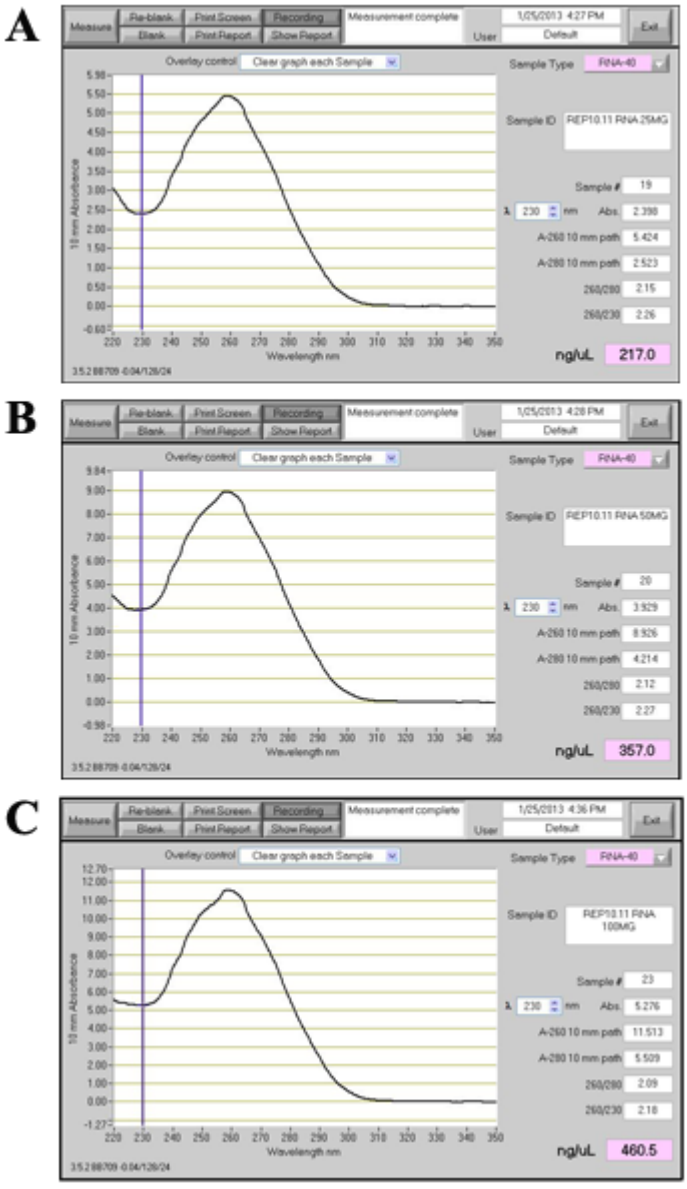

Figure 6.2. Nanodrop spectrophotometry measurements of extracted total RNA from *Ectocarpus siliculosus* strain REP10-11, measured after DNase treatment and a purification step. Total RNA extracted from REP10-11 is of high quality and free from appreciable levels of organic contaminants regardless of the biomass used in the extraction procedures. (A) 25 mg of starting biomass, (B) 50 mg of starting biomass, (C) 100 mg of starting biomass.

6.3.3. Quality and integrity of genomic DNA and total RNA

The integrity of nucleic acids was examined by 1.5 % agarose gel electrophoresis (Figure 6.3, 6.4). For co-isolated nucleic acids (Figure 6.3A), a distinct individual band of DNA and clear cytosolic and plastid ribosomal RNA bands were observed. After the purification steps, RNA intactness and the absence of DNA contamination was evident from the electrophoretic pattern that shows only cytosolic and plastid ribosomal RNA bands (Figure 6.3B). Similarly, the absence of DNA degradation is evidenced by an electrophoretic pattern showing only a distinct individual band of DNA (Figure 6.4A). These results confirm that highly purified nucleic acids were obtained, that can be used in downstream applications.

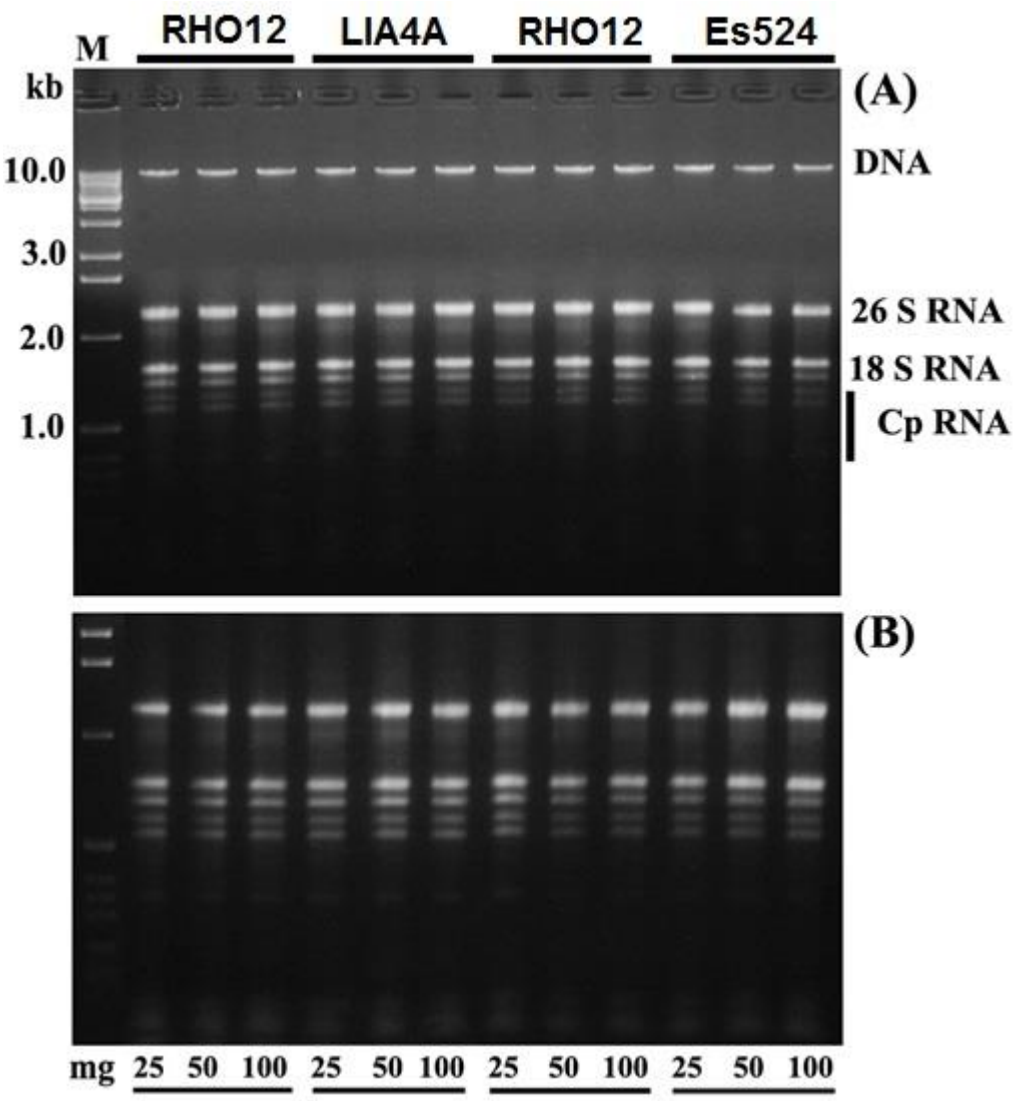

Figure 6.3. Analysis of quality and integrity of extracted nucleic acids. (**A**) Agarose (1.5% w/v) gel electrophoresis of genomic DNA and total RNA (~ 0.5 μg) isolated simultaneously from four strains of *Ectocarpus siliculosus* (RHO12; LIA4A; REP10-11; Es524), each with an initial biomass of 25, 50 and 100 mg (gel stained with ethidium bromide). DNA shows an intact single band whilst RNA shows the clear cytosolic and plastid (Cp) ribosomal bands. RNA species of low molecular weight are also apparent. (**B**) Genomic DNA contamination is effectively removed by DNase treatment, whilst the pure RNA retains intactness and quality. M: RNA Ladder, High Range (Fermentas, Italy).

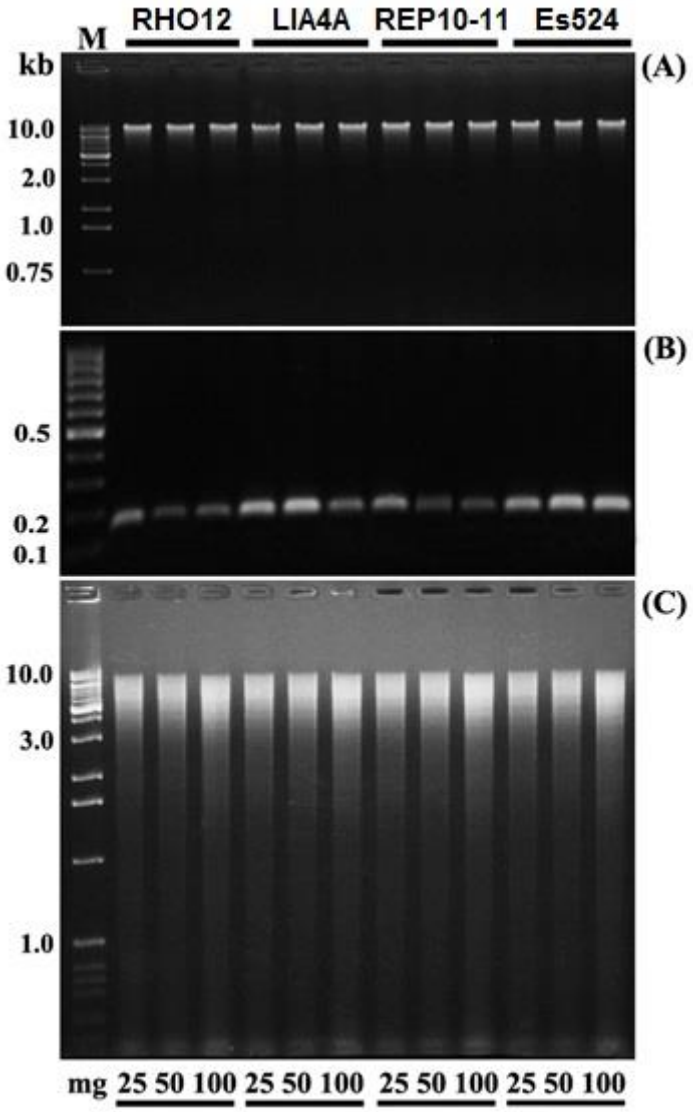

Figure 6.4. Gel electrophoresis analysis of pure DNA and its downstream application. (**A**) Agarose (1.5% w/v) gel electrophoresis of genomic DNA (~ 0.5 µg), after RNase treatment, isolated from strains of *Ectocarpus siliculosus* (RHO12, LIA4A; REP10-11; Es524) each with an initial biomass of 25, 50 and 100 mg, confirming its integrity (gel stained with ethidium bromide). (**B**) The quality of isolated DNA was confirmed by electrophoresis analysis of a DNA PCR product using an alpha tubuline housekeeping gene. (C) electrophoretic analysis of EcoRV enzyme digestion product of genomic DNA confirms that the extracted DNA is suitable for downstream application. M: 100-bp and 1-Kb DNA ladder (Fermentas, Italy).

6.3.4. Downstream applications

The quality of the genomic DNA extracted was further confirmed by results of PCR amplification and enzyme digestion performed using DNA from all strains and initial biomass quantities (Figure 6.4). In all cases, agarose gel analysis revealed that a 140-bp of the Alpha Tubulin (TUA) housekeeping gene was amplified (Figure 6.4B), and the extracted genomic DNA was successfully digested by *Eco*RV restriction enzyme (Figure 6.4C).Similarly, the intactness and quality of the obtained total RNA for downstream applications was tested through RT-PCR analysis. The total RNA obtained from all the strains selected was sufficiently pure for the successful conversion into cDNA, regardless of the amount of biomass used. Moreover, the cDNA obtained was successfully used in the amplification process, by using a specific Alpha Tubulin primer pair (Figure 6.5). This result confirms that the total RNA was of high integrity and the mRNA was intact.

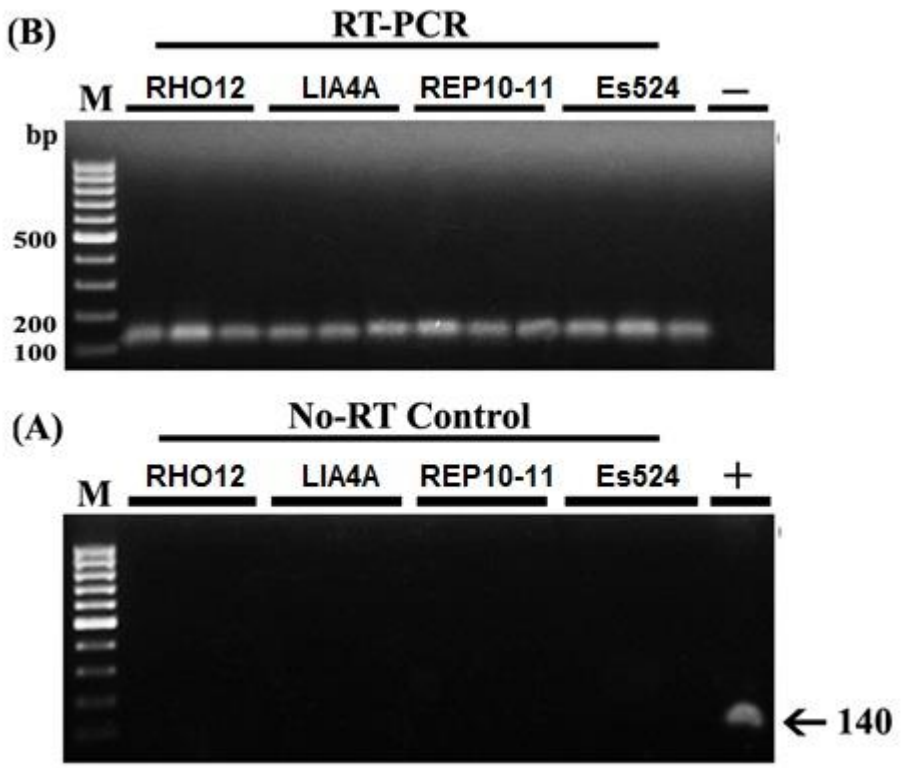

Figure 6.5. RT-PCR analysis of TUA expression of four strains of *Ectocarpus siliculosus* RNA samples extracted from 25, 50 and 100 mg of four strains of *Ectocarpus siliculosus* (RHO12, LIA4A; REP10-11; Es524) were analyzed by RT-PCR for the *Ectocarpus* alpha tubulin (TUA) housekeeping gene. M: 100-bp DNA ladder (Fermentas, Italy); −: PCR negative control (no DNA, but water was added); +: PCR positive control (no RNA but DNA was added).

6.4. Discussion

The protocol outlined in this chapter is the first to allow the co-isolation of highly pure genomic DNA and intact RNA from different strains of *E. siliculosus* using small quantities of biomass. When the method used by Le Bail et al. (2008b) was applied to isolate RNA from a single strain of *E. siliculosus* (strain Es32, CCAP 1310/4, originating from San Juna de Marcona, Peru) the yield and purity of the nucleic acids from the four strains used in this study were variable and generally poor. Moreover, when comparing this method with the one applied in Chapter III of this thesis, yields and quality of the RNA were significantly improved and followed a common procedure between strains; instead, the protocol stated in Chapter III required different purification steps between strains to obtain good yields of pure RNA. Although the effectiveness of the recently published protocol by Coelho et al. (2012a) for isolating genomic DNA from *E. siliculosus* (strain not specified) was not assessed in this study, it far exceeded the biomass used in the protocol reported here (1g *Ectocarpus*).

Obtaining high-quality nucleic acids is the primary and most critical step in molecular biology studies, particularly when using recalcitrant material such as brown algae. The presence of cell walls composed of cellulose, sulfated fucans and alginic acid (Michel et al., 2010), together with high concentrations of problematic metabolites such as lipids, polysaccharides and polyphenols that can cross-link and contaminate nucleic acids have hindered the development of an effective low-cost and time-efficient extraction protocol for brown algae. To date, different methods have been used to remove polysaccharide and phenolic contamination from plant RNA (Salzman et al., 1999; Gao et al., 2001; Azevedo et al., 2003). Extraction buffers containing high salt concentrations, such as NaCl or LiCl, have been used in the extraction of starch-rich tissues (Wallace, 1987; Vicient and Delseny, 1999), while Fang *et al*. (1992) reported that most

polysaccharides remained soluble in a high-salt (1.0–2.5 M NaCl) buffer during the ethanol precipitation step. However, such extraction buffers can result in a significant reduction of RNA yield when isolated from polysaccharides rich tissues (Singh et al., 2003). Standard RNA extraction methods using guanidine isothiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987), or RNeasy kits failed to provide satisfactory yield and purity of RNA when attempting to extract it from starch-rich tissues. Moreover, CTAB, widely used to remove contaminating polysaccharides (Murray and Thompson, 1980; Coyer et al., 1995), did not provide DNA amenable to restriction digestion when applied to green algae (LaClaire and Herrin, 1997). In agreement with this latter result, the DNA and RNA yields and purity from *E. siliculosus* samples were very low when the CTAB extraction method was used. Another critical aspect of RNA isolation is to ensure rapid and complete inactivation of RNase during the extraction. Usually, RNase can be inactivated by standard phenol/SDS extraction methods (Wilkins and Smart, 1996) but for polysaccharide-rich tissues such an approach cannot be used successfully due to the binding of polyphenolic compounds to nucleic acids that results in co-precipitation of nucleic acids with starch and proteins (Wilkins and Smart, 1996; Wang et al., 2005; Hoarau et al., 2007).

The protocol reported in this chapter is rapid, relatively non-toxic, inexpensive, and applicable for extracting large quantities of high purity DNA and RNA from small amounts (25 mg) of biomass of *E. siliculosus* strains isolated from different environmental conditions. It employs a high pH (pH 9.5) extraction buffer, containing Tris-HCl (100 mM) and sarkosyl (1%), which ensures maximum solubility of nucleic acids, and removes most polysaccharides and other insoluble materials from polysaccharide-rich tissues. Oxidative cross-linking of nucleic acids by phenolic compounds was avoided by using DTT (5mM) in the extraction buffer; it also has the additional advantage of inactivating

RNases during the homogenization and extraction steps (Pearson et al., 2006). The method uses ethanol and potassium acetate simultaneously which aids precipitation of polysaccharides in brown algae (Gao et al., 2001). Sodium acetate was used along with isopropanol for precipitation to enhance the DNA/RNA yield and shorten the precipitation time. A phenol:chlorophorm:isoamyl alcohol step was subsequently used to purify the nucleic acids.

The highest yield of total DNA and RNA (0.284 and 0.195 μg mg^{-1} fresh weight respectively) was obtained from a biomass of 25 mg. These data are comparable with those for other brown algae such as *Fucus vesiculosus* and *Saccharina japonica* but with the advantage that much smaller quantities of material were used (Su and Gibor, 1988; Cseke et al., 2004); for example, 250 and 500 mg of biomass are required for good yields of nucleic acids from *F. vesiculosus* and *L. japonica.*

The purity of nucleic acids was confirmed by the A_{260}/A_{280} ratio which indicates that both DNA and the RNA samples were pure and effectively separated from protein and polysaccharides. In general, for the four strains used, the highest level of purity was obtained from 25 mg, followed by 50- and 100-mg biomass. Therefore, it is strongly recommended to use small quantities of starting material for nucleic acids isolation from brown algae. The integrity of nucleic acid samples was examined on a 1.5% agarose gel; all RNA samples were intact as judged by the sharp and distinct cytosolic and plastid ribosomal bands in the agarose gel. Moreover, agarose gel electrophoresis showed a distinct individual band of intact genomic DNA.

The RNA was suitable for RT-PCR and could be used in sensitive downstream applications such as real-time qRT-PCR assays and next-generation sequencing

technologies. In addition, genomic DNA was of good quality, free of interfering compounds and suitable for PCR. It would also be suitable for Southern blot hybridization, as it produced reliable restriction enzyme digestion patterns, whole genome methylation sequencing and DNA sequencing.

In conclusion, a protocol for extracting high-quality DNA and DNA-free RNA from small samples of the model brown alga *E. siliculosus* has been developed that should expedite studies aimed at understanding biological functions of brown seaweeds, an ecologically and economically important group of coastal and estuarine photoautotrophs from cold and temperate latitudes. Furthermore, it is suggested that it could be used effectively to extract nucleic acids from a range of other algal species that have polyphenol- and polysaccharide-rich tissues.

Chapter VII

General Discussion

With an emphasis in intra-specific responses, we have conducted a complete investigation of the mechanisms against Cu stress, and more generally to metal stress (field experiments), in different strains of the model brown alga *Ectocarpus siliculosus*. We have carried out a holistic evaluation at different levels of biological organization, which comprised physiological, biochemical and molecular responses, such as effects on growth, metal exclusion mechanisms, the production of metal chelators, and a complete description of reactive oxygen metabolism. To be able to accomplish these measurements, several published protocols had to be applied and adapted to *E. siliculosus*, and sometimes specifically to certain strains. The latter particularly associated to our molecular analyses, which derived in developing our own protocol for the extraction of nucleic acids. In this last Chapter VI, we will provide an overview of our findings and discuss the general implications of the investigation to improve our understanding of metal-stress metabolism and, furthermore, to increase our knowledge about inter-population responses against metals in brown algae.

In Chapter III we described our Cu exposure laboratory experiments, conducted on the strains Es524 and LIA4A, with origin in Chile (Cu polluted location) and Scotland (pristine site), respectively. We exposed these strains to a range of Cu concentrations ranging between 0 and 2.4 µM for 10 days. We observed that the relative growth rates were significantly lower in LIA4A than in Es524 at the highest levels of Cu exposure (section 3.3.1). This information suggests that Cu stress is causing a strong disruption, especially in the metabolism of LIA4A, and therefore significantly affecting its physiology and development.

Interesting patterns were also observed in metal accumulation, especially when comparing the proportion between intracellular and extracellular-adsorbed Cu in Es524

and LIA4A (section 3.3.2). In both strains the total Cu accumulation increased in parallel with growing Cu exposure; however, the percentage of intracellular Cu (within total accumulated) in LIA4A at the two highest levels of Cu exposure were greater in LIA4A than in Es524. Moreover, under exposure to 1.6 and 2.4 µM Cu, we could observe that the levels of exchangeable (extracellular) Cu were much higher in Es524 in relation LIA4A. Hall et al. (1979), after conducting Cu exposure to different strains (resistant and non-resistant) of *E. siliculosus*, concluded that Cu exclusion was mainly associated with the permeability of the cell membrane. Our findings might refuse this hypothesis and suggest that, in addition to the cell membrane, Cu exclusion is also dependent on extracellular metal chelation. The latter might be related to different factors, such as exudation of metal chelators, efficient symbiosis with epiphytic bacteria, and the availability of binding sites in the cell wall; experiments with axenic cultures might be helpful to fully elucidate the mechanisms of action. Moreover, our results show that these exclusion mechanisms can be highly variable between populations, and are likely to be inherited due to the known origin of these strains.

The expression of the genes encoding the enzymes GCL, GS and PCS, in the pathway of production of GSH-PCs were also highly strain-specific (section 3.3.3). LIA4A displayed an upregulation of *GCL1* and *GCL2* until 0.8 µM, and downregulation at higher levels of Cu, whereas in Es524 there was a clear pattern of upregulation with growing Cu exposure showing the highest levels of transcripts at 2.4 µM Cu. Surprisingly, the highest levels of *GS* expression were observed at control condition and under 0.8 µM Cu; however, beyond this Cu exposure there was a downregulation of *GS* in LIA4A. In case of Es524, there was a clear pattern of increase in *GS* transcripts with the highest levels of expression at 1.6 and 2.4 µM Cu. Finally, patterns for *PCS* were similar to those described in GCL genes, with the strongest induction in Es524 at 2.4 µM Cu (1500 fold from

control). The data strongly suggested that strain Es524 was better prepared than LIA4A to synthesise GSH and PCs under these levels of Cu exposure.

Content of total glutathione (GSH + GSSG) increased for Es524 and LIA4A, with no clear patterns between them. This information was hard to link to the expression of *GLC* and *GS*, which were more expressed in Es524 than in LIA4A under high Cu exposure. However, as we later describe in Chapter VI, even though total glutathione was similar between strains, the proportion of GSSG in relation to GSH was notably higher in LIA4A than in Es524 at the highest levels of Cu exposure; the later a clear sign of higher oxidative stress in LIA4A. On the other hand, the content and level of polymerization of PCs were clear evidence of Cu resistance in the strains assessed (section 3.3.4). The oligomers PC2 were observed in both Es524 and LIA4A; however, only in case of Es524 content of PC2 increased significantly in parallel with growing Cu exposure. Moreover, longer chain PC3 were only identified in Es524, and not in LIA4A. Although there were no patterns of increase of PC3 in Es524 up to the levels of Cu exposure used in this investigation, just their presence suggests that this strain is better prepared to counteract the effects of Cu stress. Additional experiments at realistic higher levels of Cu might help disclosing if PC3, or even longer chain PCs, are induced in Es524 and even in LIA4A. This is the first investigation that assessed the effects of chronic Cu exposure on the mechanisms mediating GSH and PCs synthesis in brown algae, and highlighted these as an active defence system to counteract Cu toxicity in their cells. Moreover, we provide evidence indicating that these mechanisms can be population-specific, and due to the origin of Es524 and LIA4A, further support the hypothesis that they have been inherited.

In Chapter IV, we conducted similar experiments to those described in Chapter III, but we measured different parameters associated to oxidative stress responses, content of

antioxidants, and the activity of antioxidant enzymes. In addition to Es524 and LIA4A, we incorporated the strain REP10-11 to the study, sampled in a metal polluted (including Cu) location in England. While strain LIA4A showed a notable increase in the levels of the H_2O_2 and lipid peroxidation with increasing Cu exposure up to 2.4 µM (section 4.3.1), the other strains (especially Es524) were less affected by Cu exposure. Similarly, when we measured pigments (section 4.3.1), the strain LIA4A showed the highest Cu-mediated stress as manifested in Chl*a*/Chl*c* and fucoxanthin degradation. An interesting point of this study was that, only inthe strain Es524, we observed a clear pattern of increase in the levels of fucoxanthin. The results suggests that fucoxanthin can be having antioxidant properties in the chloroplast of Es524, which would be in agreement with the antioxidant functions of other xanthophyll pigments in different photosynthetic organisms (Dall'Osto et al., 2010); however, this assumption needs to be experimentally tested. This information can be highly relevant, not only as description of a new antioxidant for brown algae, but also because it would provide more evidence of inherited adaptations.

In terms of the levels of antioxidant compounds, the differences were also evident between strains and in agreement with their original exposure backgrounds. While the levels of total glutathione, ascorbate and phenolics increased for all the strains assessed with increasing Cu exposure, the main differences between strains were observable when identifying relations in reduced and oxidised forms of glutathione (GSH-GSSG) and ascorbate (Asc-DHA) (see section 4.3.2). It appears that Es524 and REP10-11 have developed efficient mechanisms in terms of glutathione recycling, which were manifested by a pattern of increase in GSH levels with greater Cu exposure; however; GSH:GSSG ratios revealed that glutathione recycling was more efficient in REP10-11. Likewise, Asc levels were greater for both REP10-11 and Es524 in parallel with increasing Cu concentrations; moreover, although DHA levels increased for both strains from polluted

locations with higher Cu exposure, they were similar to Asc content (especially in REP10-11), suggesting efficient Asc recycling. The strain LIA4A, instead, displayed several signs of saturation, given by a decrease in the levels of GSH and Asc, and an over-increase in GSSG and DHA, above 0.8 µM Cu. The gathered information suggests that REP10-11 has the most efficient glutathione-ascorbate interactions in situations of Cu excess, followed by Es524, and finally LIA4A. The activity of the antioxidant enzymes was also evidence of differential response between strains. The activity of SOD was the highest in Es524 when exposed to Cu, followed by REP10-11, and the lowest in LIA4A (section 4.3.3). Another interesting point of our investigation was that we found that only the strain Es524 was capable of increasing the activity of the peroxidase enzymes CAT and APx together, while REP10-11 and LIA4A could increase Apx, but not CAT. These results were also in agreement with the content of H_2O_2, which was observed to be the highest in LIA4A under Cu exposure. Different investigations have provided evidence suggesting that APx and CAT can display species-specific patterns in their joint activity against H_2O_2 (Smirnoff, 2005a; Contreras et al., 2009; Cansev et al., 2011); however, our results provide evidence suggesting that these patterns can also be population-specific, and taking into account the origin of the strains, likely to be inherited. The results in Chapter VI give us relevant evidence showing that, although antioxidant defences following metal exposure can be variable between brown algae populations, the antioxidant mechanisms can be highly divergent, even between populations with origin in metal polluted locations.

The experiments and measurements carried out in Chapters III and IV of this Thesis evidenced clear patterns between strains of *E. siliculosus*; most of them in agreement with the history of Cu exposure they were subject to in their original environments. The strain LIA4A, originating from a pristine location of Scotland, displayed several signs of saturation under Cu stress that demonstrated its low resistance.

In contrast, the strains Es524 and REP10-11, from Cu polluted locations in Chile and England, respectively, showed to be less affected by Cu-mediated oxidative damage, and evidenced strong defence mechanisms against Cu exposure. We found that Es524 had strong Cu exclusion mechanisms, with higher Cu accumulation in the cell wall, and lower intracellular concentrations of Cu, in relation to LIA4A. When exclusion mechanisms were insufficient and Cu concentrations became a threat intracellularly, Es524 was more capable than LIA4A of increasing the levels of all the metal chelators measured: PCs, glutathione, and phenolics (similarly with REP10-11 but no PCs were measured for this strain). In terms of oxidative stress responses and damage, the strain LIA4A was always the most affected, followed by REP10-11, and finally Es524 (the least affected). An important finding of our investigation was that even within a 'tolerant population' of *E. siliculosus*, the strategies to cope with Cu-induced oxidative stress were clearly different. For example, Es524 seems to have good levels of antioxidants, but more importantly, a strong induction of antioxidant enzymes. On the other hand, REP10-11 appeared to base its antioxidant defences on efficient glutathione-ascorbate interaction, which has been recognised as one (if not the most) important antioxidant mechanism of the cell (Noctor and Foyer, 1998; Smirnoff, 2005a; Noctor et al., 2012). To the extent of our knowledge, our investigation is the first to provide such a complete description of metal defences and the reactive oxygen metabolism in different populations of brown algae, which also helps elucidating the basis behind intra-specific metal resistance in these organisms. It appears that metal resistance and the development of ecotypes in resistance in brown algae is importantly related to genomic and epigenetic adaptation to metal stress; further molecular studies in this direction will further complete the puzzle.

Brown seaweeds have become popular organisms in the field of ecotoxicology, and have been commonly used for over 60 years as biomonitors of metal stress; the latter arises mainly because of the metal resistance of some of their species and their high metal accumulation capacity (Brown and Depledge, 1998; Sáez et al., 2012a). Most of the metal biomonitoring efforts using brown algal species have been carried out with a 'passive' approach; thalli of local populations are sampled and different physiological, chemical, biochemical, or molecular analyses are conducted. However, as also demonstrated during this thesis, brown algae can develop resistant populations; differential metal stress responses between populations can bring uncertainties to the assessment and induce false environmental diagnosis. Several authors have suggested that 'active' biomonitoring can be a better option; seaweeds of the same population are cultured in the laboratory and then introduced into locations of interest (see section 5.1 for further information). However, physiologically complex seaweeds such as kelps have usually been used; the latter can be time-consuming and logistically complicated due to their large size and complex physiology. To overcome these issues we designed a novel 'active' biomonitoring method with *E. siliculosus* using oxidative stress biomarkers; to validate the protocol and decrease chances of error induced by inter-population variations in response, two strains with different pollution backgrounds were used; Es524 from a Cu polluted location in northern Chile (same as used in laboratory experiments), and the strain Es147 from a low Cu polluted location of northern Chile. *E. siliculosus* was placed inside dialysis membrane and protected with a plastic bottle with holes; the device was deployed in a metal polluted and a pristine location of central Chile for 10 days. We measure similar parameters as those assessed in Chapter III and IV, related to oxidative stress responses and total metal accumulation, respectively.

In terms of total metal accumulation, both strains presented similar patterns. Higher accumulation of metals Cu, Al, Pb, and Fe, was observed for both Es524 and Es147 in Ventanas (the polluted location), in relation to Quintay (the pristine control) (section 5.3.1). Metal Cd, however, presented the opposite trend, with higher levels in Quintay in comparison to Ventanas, for both strains; although these results were in agreement with past records of Cd and were attributed to the strong upwelling phenomena occurring in close proximity to Quintay.

While strain Es524 did not show differences in oxidative responses between Quintay and Ventanas, Es147 showed clear signs of saturation given by higher levels of H_2O_2, lipid peroxidation, and lower levels of Chl*a* and Chl*c* in in Quintay, compared to Ventanas (section 5.3.2). Moreover, as observed in the laboratory experiments in section 3.3.1, fucoxanthin was only significantly higher in Es524, but not in Es147, in Ventanas. In relation to the levels of antioxidants, Es524 and Es147 displayed similar trends between locations assessed. Levels of total glutathione and ascorbate were higher in Ventanas than in Quintay for both strains; relations between reduced and oxidized forms of these compounds (GSH-GSSG/Asc-DHA) were also similar between strains and showed higher oxidation in Ventanas than Quintay (see section 5.3.3). Content of phenolic compounds were also similar between Es524 and Es147, with higher levels in Ventanas than Quintay (section 5.3.3). In terms of the activity of the antioxidant enzymes measured, only the strain Es524 was capable of increasing the activity of all antioxidant enzymes (SOD, APx, CAT, and GR) in Ventanas, in relation to Quintay, whereas Es147 was capable of just increasing the activity of SOD (section 5.3.4). Although the protocol was successful in representing metal bioavailability and dose in the locations assessed, as in the laboratory experiments, Es524 and Es147 presented a few differences. To avoid errors induced by differential stress responses, only those biomarkers that behaved similarly between strains

were recommended for future environmental assessments, namely: metal accumulation, glutathione and ascorbate in reduced and oxidised forms, levels of phenolic compounds, and the activity of SOD. The method can be applied globally throughout temperate regions, even in the absence of *E. siliculosus* populations and rocky/hard substrata, it is cost-effective, and the results can reliably represent the bioavailable metal status of the environment.

Another interesting point of our investigation was the fact that there was congruency in the stress responses observed in the laboratory and those in the field. The strain Es524 seemed to have sufficient resistance in order to cope with a Cu enriched environment or when transplanted to a metal-polluted location, whereas other strains such as LIA4A and Es147 presented clear signs of metal-induced stress and damage. We observed that the levels of fucoxanthin were significantly higher than controls under both laboratory and field experiments for the strain Es524, supporting even more the hypothesis that this xanthophyll might be acting as an antioxidant; furthermore, this pattern appears to be highly strain-specific and only observable in Es524, but not in REP10-11 which is also considered a resistant strain. The levels of glutathione and ascorbate and redox states were also similar in Es524 between both experimental approaches; also similar between REP10-11 (laboratory) and Es147 (field). In terms of the antioxidant enzymes, Es524 was successful in increasing the activity of all of them under Cu exposure in the laboratory (SOD, APx, and CAT), and also in the polluted location in field experiments (SOD, APx, CAT, and GR). On the other hand, the other strains assessed were only capable of increasing the activities of certain antioxidant enzymes. We have demonstrated that in addition to the known species-specific patterns in the collaboration of peroxidase enzymes such as APx and CAT, these trends can be also population-specific and most likely inherited.

Development of appropriate biomarkers requires an understanding of the cellular and physiological processes associated with cell injury, molecular damage and impairment of protective systems as well as how they are manifested as reductions in reproductive output, competitive ability and survival of individuals. This understanding could be gained using a combination of laboratory-based investigations and field studies. Laboratory experiments help identifying specific metabolic responses; however, the information can be hardly extrapolated to the natural, more complex, environment. On the other hand, field experiments lack mechanistic information in order to attribute responsibility to particular stressors. To the extent of our knowledge, our investigation is novel in combining both approaches, and has proved that similar effects can be observed under metal exposure in the laboratory and in the field. This information is especially useful for biomonitoring purposes; we have observed that certain parameters associated with metal accumulation and the reactive oxygen metabolism can behave similarly between strains or populations of *E. siliculosus,* even with different metal-exposure backgrounds, supporting that the tested responses in the field are, in effect, a reaction to metal stress.

As a result of experimenting with different strains of *E. siliculosus* for subsequent qRT-PCR analyses, we tested several published methods for RNA extraction (see section 6.1). Differently from vascular plants, extraction of high quality nucleic acids from brown algae is a complex procedure due to their high content of interfering compounds, such as polysaccharides, starch and polyphenols (Chomczynski and Sacchi, 1987). Most of the methods available required big quantities of *E. siliculosus* (see Le Bail et al., 2008b; Coelho et al., 2012a), which is a problem when working with this species due to its small size and low biomass production. On the other hand, we observed that with existent protocols the yields and quality of RNA were significantly different between strains. To

perform transcriptomic characterization of any organism, for analyses such as qRT-PCR, microarray, or RNA-seq, the RNA must be as pure and free from DNA. The most commonly used method for RNA extraction from *E. siliculosus* was published by Le Bail et al. (2008b), which uses a CTAB-based extraction buffer. The latter protocol was tested but we obtained low yields and poor quality RNA from small quantities of *E. siliculosus* biomass; moreover, quantity and purity of RNA was highly variable between strains. To overcome these issues, we designed a new method, based in a Tris-HCl extraction buffer with high pH (9.5). During the first trials, before purification of RNA, we observed that the DNA yields were also high; therefore, alternatively we also applied a parallel DNA isolation. The protocol was assessed in different strains, from biomass of 25-, 50-, and 100-mg. The method was successful in extracting good quantities of pure RNA/DNA from all the strains assessed, significantly more efficient than the protocol published in Le Bail et al. (1998). Our method produced good yields of high quality nucleic acids from only 25 mg of biomass; in average, 0.195 and 0.284 μg mg^{-1} fresh weigh of RNA and DNA, respectively. The overall quality of nucleic acids was confirmed by A260/A280 ratio and agarose gel electrophoresis. The isolated RNA can be used directly in downstream applications such as RT-PCR. Furthermore, we have recently conducted RNA extraction with this method for RNA-seq in experiments conducted on Es524 and LIA4A; we have received results from sequencing and quality scores validate that the mRNA was highly pure and preserved (next paragraph for description of experiments). Finally, the genomic DNA obtained with our extraction protocol was suitable for PCR, producing reliable restriction enzyme digestion patterns. Co-isolation of DNA/RNA from different strains shows that this method is likely to be widely applicable for intra- and inter-specific studies not only on *E. siliculosus*, but in other recalcitrant algae.

Our results at different levels of biological organization on different strains of *E. siliculosus* after metal stress, suggest that a long history of Cu exposure can mediate the development of inherited resistance in brown algae. As we observed in the results from gene expression of the enzymes in the pathway of production of GSH and PCs, those defenses start at least at the level of transcripts (section 3.3.3). To underline the molecular basis of metal stress metabolism in brown algae, I am currently conducting RNA-seq experiments in the two most responsive strains of *E. siliculosus* assessed during this Thesis, Es524 (Cu polluted location) and LIA4A (pristine location); experiments were conducted as described in Chapters III and IV, but only at control conditions and under 2.4 µM Cu exposure. RNA-seq is the latest approach available for studying gene expression at the level of the whole transcriptome, using Next Generation Sequencing (NGS) technologies (Maher et al., 2009). RNA-seq produces a large amount of sequence reads that need to be stored, retrieved and processed using a series of bioinformatics methods and software. Parameters such as quality of bases and reads, mapping/alignment of reads, identify exon junctions and poly (A) ends, RNA editing, structure of transcripts, and quantification of expression signals, are currently being assessed. These experiments will significantly help us to understand genomic and transcriptomic adaptations in different populations in brown algal species, for example: determining genomic variants between strains and exposures that might help increasing the effectiveness in the action of defence proteins; assessing the importance of families or specific genes in Cu stress metabolism, and compare them between strains; and contribute to our understanding on contribution of the different metabolic defences known in brown algae against metal stress, extra- and intra-cellular, and determine related intra-specific differences in response.

Appendices

Appendix I

Preliminary assay for PCs production and characterization in *Ectocarpus siliculosus* through Cd-induction

In order to verify presence and molecular structure of PCs in *Ectocarpus siliculosus* one culture of Es524 strain was exposed for seven days to Cd (189 nM Cd^{2+}), the strongest PCs-inducer (Gledhill et al., 2012). Results showed (Figure 3.1) that Es524 control cultures had a constitutive low level of PCs present only as dimers (PC2) and trimers (PC3). Under Cd exposure the production of different chain length PCs were significantly induced with respect to control conditions ($p < 0.001$) although the shorter chain PC2 and PC3 were the predominant oligomers compared to the tetramer PC4. Total glutathione content was not significantly different ($p > 0.05$) between control and Cd-treated cultures.

As commonly observed the fluorescence chromatogram for Cd exposed *E. siliculosus* produced many peaks (Figure 3.2), making identification of PCs challenging. Direct analysis of GSH and PCs by ESI-MS post fluorescence detection allowed for unambiguous identification of thiols in our samples and confirmed the molecular structures of the derivatised PCs oligomers (PC2, PC3, PC4) for the first time. Resultant MS2 fragments corresponding to GSH, the dimer (PC2), trimer (PC3) and tetramer (PC4) of PCs are presented in Figure 3.2 of the Thesis, panels B-E. For derivatised GSH and PC2, fragmentation occurred via the loss of the terminal NH3 (Δ17) and carboxylic acid group (Δ44) of γ-glutamic acid to produce the product ions m/z 435 and m/z 857, respectively. For PC2 and PC3, losses of 2-amino acetic acid (Δ75) from glycine were observed, producing product ions of m/z 845 and m/z 1267, respectively. Fragmentation of derivatised PC2 and PC3 also produced ions corresponding to the derivatised lower order peptide (m/z = 498 and m/z = 920 in MS2 spectra of PC2 and PC3, respectively).

Concentrations of PC4 in the Cd exposed sample were very low, so that fewer fragmentation scans (n=3) were obtained and results are therefore less robust. However, initial fragmentation of PC4 appeared to involve the loss of $C_2H_5NO_2$ from γ-glutamic acid to produce m/z 1677.

Appendix II

Details about materials and the procedure conducted for the extraction and purification of nucleic acids.

1. Materials

1.1. Consumables

- 1.5 - 2.0 mL microcentrifuge tubes DNase- and RNase-free (both tubes have ultrasmooth conical bottoms to allow 100% visibility) (e.g. Sigma Aldrich or Eppendorf);
- 15 mL Corning centrifuge tubes DNase- and RNase-free;
- 3 mm solid-glass beads **(**Sigma Aldrich Z143944);
- Laboratory goggles;
- Micropipette (e.g. Gilson or Eppendorf);
- Pestle Mixer RNase-free, used for grinding soft tissue in microcentrifuge tubes (e.g. Krackeler Scientific, Inc. (186-749520-0090)).

NOTE: When alternative plastic-ware was used, iit was made for that it is resistant to phenol and chloroform.

1.2. Solutions and reagents

- RNA Extraction Buffer (EB): 100 mM Tris-HCl, pH 9.5; 150 mM NaCl; 1.0% sarkosyl. Add 5 mM DTT before use;

- 100% and 75% (v/v) ethanol made with DEPC-treated double-distilled (dd) H2O; (Sigma-Aldrich, Cat. No 02860);
- 1 M Dithiothreitol (DTT) (Sigma-Aldrich, Cat. No 646563);
- 2-mercaptoethanol (Sigma-Aldrich, Cat. No M3148);
- Chloroform (Sigma-Aldrich, Cat. No 25690);
- DEPC-treated 3 M sodium acetate (pH 5.2);
- DEPC-treated 3 M potassium acetate (pH 4.8);
- Diethylprocarbonate (DEPC) (Sigma-Aldrich, Cat. No D5758). CAUTION:DEPC is a carcinogen, work under fume hood;
- DNase I recombinant, RNase free enzyme (Roche Diagnostic, Cat. No 04716728001);
- Isoamyl alcohol (3 Methyl-1 Butanol, 99%), (Sigma-Aldrich, Cat. No W205710);
- Isopropanol (2-propanol) (Sigma-Aldrich, Cat. No I9516);
- Liquid nitrogen;
- Phenol solution pH 8.0 (Sigma-Aldrich, Cat. No P4557);
- Ribonuclease A enzyme (Sigma-Aldrich, Cat. No R4875);
- RNAse-free water. Add 1 ml l^{-1} DEPC, shake the solution and incubate overnight under a fume hood. Autoclave properly to inactivate remaining DEPC.

1.3. For agarose gel electrophoresis analysis

- Agarose (Roche Cat. No 11388991001);
- Ethidium bromide (Sigma-Aldrich, Cat. No E7637);
- TBE 5X concentrate (Sigma-Aldrich, Cat. No 93306). TBE was prepared in RNase-free water to reach the TBE 0.5X working solution;
- RNA ladder High Range (Fermentas, Cat. No SM0423);

- DNA ladder Gene Ruler ^{TM}, 100 bp and 1kb (Fermentas, Cat. No SM0241, SM0312).

1.4. Equipment

- Autoclave;
- Benchtop laboratory shaker (e.g. INNOVA® 2000 – 2050);
- DNA electrophoresis equipment;
- Eppendorf Minispin (e.g. Eppendorf Plus w Rotor Nice);
- Fume cupboard for handling phenol, chloroform, DTT and 2-mercaptoethanol;
- NanoDrop ND-1000 Spectrophotometer;
- Refrigerated laboratory centrifuge (e.g. Eppendorf 5702 R);
- Standard laboratory instruments (beakers, cylinders, flasks, magnetic stirrer, tweezers etc);
- Thermocycler (e.g. Eppendorf);
- Thermomixer (e.g. Eppendorf);
- Vortex (e.g. Genie® 2 (S-7350-1));

Note: Glassware and pestles were cleaned with detergent, filled with 0.1% DEPC, incubated at 37°C overnight, and then autoclaved for 30 minutes.

2. Methods

Steps of the RNA-DNA method will be described in the following 3 sections: General information (2.1), Nucleic acids isolation (Section 2.2), and Purification step (Section 2.3).

2.1. General information

- For DNA extraction, sterilized tips were used, tubes and solutions;
- For RNA extraction, RNase-free tips, tubes and any other consumables were used. Moreover, RNase free/DEPC-treated solution throughout were used. All the equipment were well cleaned, a surface decontaminant solution that destroys RNases was also used; they were ideal for cleaning work surfaces, pipettors, and equipment that must be RNase-free (e.g. Ambion® RNaseZap®).

2.2. Nucleic acid isolation (Figure 1)

2.2.1. Before starting

(1) Sufficient volume of stock solutions was prepared: 1M Tris-HCl (pH 9.5), 1M DTT, 3M potassium acetate (pH 4.8), 3M sodium acetate (pH 5.2), 5M NaCl, 10% sarkosyl, 100% ethanol.

(2) Isopropanol, 75% and 100% ethanol at -20ºC. Solutions 1M Tris-HCl (pH 9.5), 3M potassium acetate (pH 4.8), 3M sodium acetate (pH 5.2), 5M NaCl, (24:1, v/v) chloroform:isoamyl alcohol, phenol, 10% sarkosyl and 1M DTT was kept at 4ºC.

2.2.2. Tissue harvesting

Genomic DNA and total RNA were extracted from four strains of *Ectocarpus siliculosus* (RHO12; LIA4A; Es524; REP10-11), isolated from metal-polluted and pristine sites in different geographical locations as described in Table 1.

Table 1. Geographical origin and environmental conditions of strains used

Strain	**Geographic location**	**Site**
RHO12	Wales	Pristine
LIA4A	Scotland	Pristine
Es524	Chile	Metal polluted
REP10-11	England	Metal polluted

(3) 2 mL microcentrifuge tubes were prepared.

(4) On dry ice, 25 to 100 mg of fresh material were harvested directly into 2 mL microcentrifuge tubes and freeze immediately in liquid nitrogen.

WARNING: Do not overfill tubes and ensure algal material is free of surface water for optimal tissue homogenisation. To obtain the best quality nucleic acids it is essential that harvested material is frozen rapidly and that the material is not allowed to thaw. At this stage, material can be stored at -80°C for several weeks before extraction.

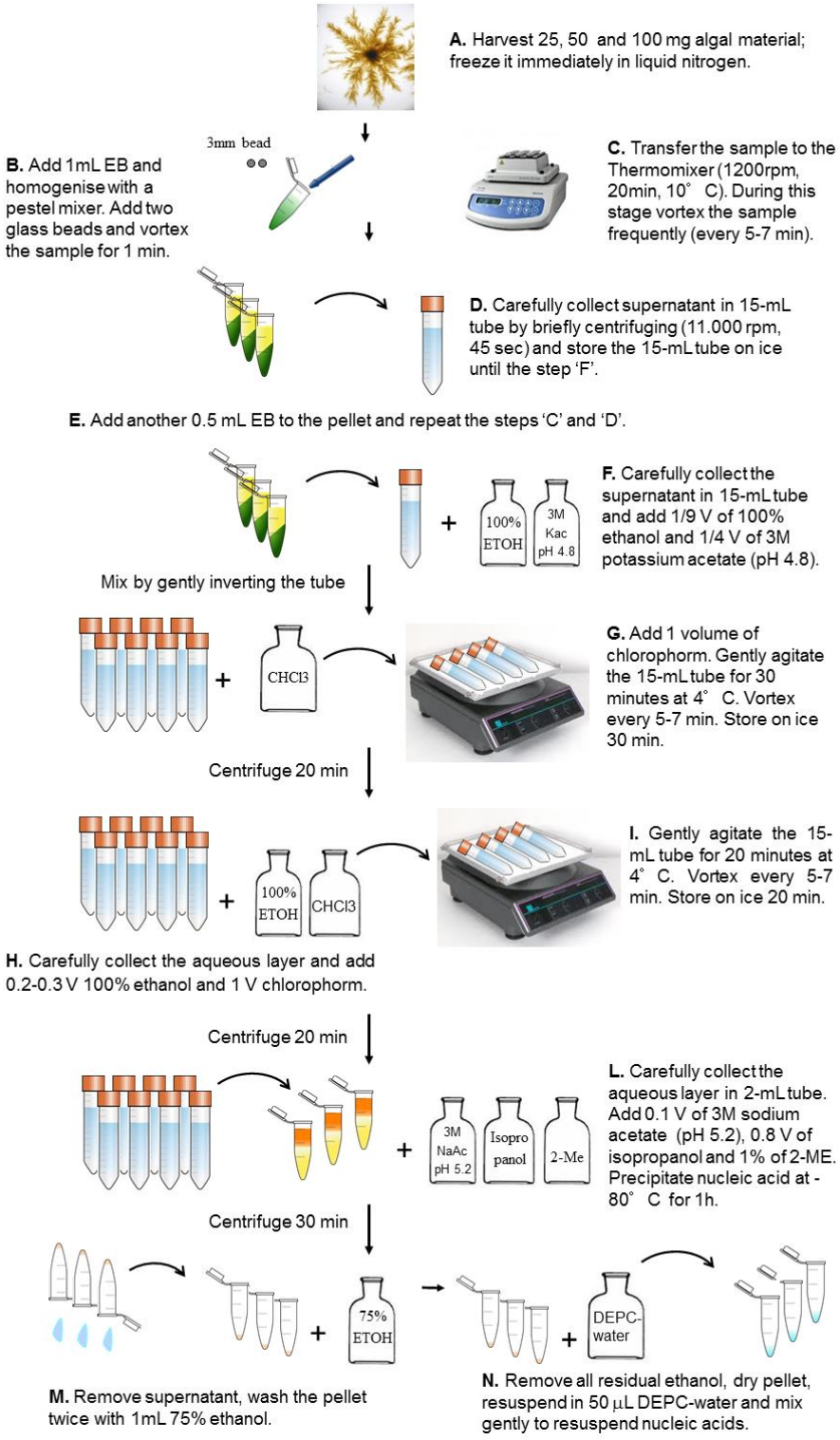

Figure 1. Summary of the extraction protocol for nucleic acids from *E. siliculosus.*

2.3. Step by step protocol (Timing: 7.5 hours)

2.3.1. Cell lysis, inactivation of cellular nucleases and separation of nucleic acid from cell debris (Timing: 1 hour)

All these steps, including centrifugation, was carried out under cold conditions (e.g. on ice or in a cold-room). Fume hood and wear personal protective equipment was used when handling 2-mercaptoethanol, DTT, phenol and chloroform. Always wear clean gloves and change gloves frequently.

(5) Extraction Buffer (EB) was prepared (Table 2). Prior to starting the extraction, 5 mM DTT were added. Once these were added the shelf-life of the buffer was only 2-3 days;

Table 2. Extraction Buffer (EB) guideline

Extraction Buffer	**N° Samples**	**1M Tris-HCl**	**10% Sarkosyl**	**5M NaCl**	**Nuclease-free water**	**1M DTT**
10 mL	6	1 mL	1 mL	0.3 mL	7.65 mL	50 µL
25 mL	16	2.5 mL	2.5 mL	0.75 mL	19.12 mL	125 µL
50 mL	32	5 mL	5 mL	1.5 mL	38.25 mL	250 µL

(6) 1 mL of EB to each collection tube of frozen algae was added and with a blue pestle mixer the tissue was homogenized until the mixture thawed (about 1 min). New pestles were used for different samples.

(7) Two 3 mm solid-glass beads were added to each tube and the content mixed vigorously using a vortex for 1 min. When processing multiple samples, the sample was left on ice while carrying out steps 6 and 7 on other samples.

NOTE: In this protocol, samples were not ground in liquid nitrogen to obtain a fine powder but the algae were homogenised directly as described in steps 6 and 7.

(8) The samples were transferred to the thermomixer; the samples were mixed and shaked at 1200 rpm for 20 min at 10°C. To aid effective tissue homogenisation, samples were vortexed every 5 min.

(9) The samples were centrifuged for 45 sec at 11,000 rpm in the Eppendorf Minispin.

(10) The supernatant containing nucleic acids was collected and transferred to a 15 mL tube. The tube was kept in ice until step 13.

(11) he extraction step was repeated by adding additional 0.5 mL EB in the 2 mL microcentrifuge tube containing both the pellet and the 3 mm solid-glass beads, and shaked vigorously for 1 min. The tube was kept on ice if processing multiple samples.

(12) Steps 8 and 9 were repeated.

(13) Supernatant was added to the 15 mL tube previously used in step 10 to obtain a final volume of 1.5 mL of extract.

2.3.2. Removal of proteins and organic contaminants (Timing: 2-2.5 hours)

(14) The following reagent were slowly mixed in the indicated order (Table 3). Tubes were inverted 8-10 times.

NOTE: Simultaneous presence of absolute ethanol and potassium acetate aids polysaccharides precipitation (Su and Gibor, 1988).

Table 3. Reagent used to remove contaminants

1V (Volume) Supernatant
1/9 V of absolute ethanol (pre-cooled)
1/4 V of 3M potassium acetate, (4.8 pH) (pre-cooled)

(15) 2 mL of chloroform:isoamyl alcohol were added (24:1, v/v) and the tube shaked vigorously for 1 min. This step allowed separation of nucleic acids from the mixture.

WARNING: Change gloves immediately if you spill chloroform on them. Avoid dripping chloroform into the tubes; due to its low viscosity chloroform drips out of the tip and could make the label peel off of the tube.

(16) Using a bench-top shaker, 15 mL tube was gently mixed for 30 min at 4°C. Sample were vortexed every 5-7 min during shaking.

(17) The tubes were incubated upright on ice for 30 min.

(18) The sample was centrifuged at 11,000 rpm for 20 min at 4°C in order to separate the organic phase from the aqueous phase.

(19) Upper aqueous phase was carefully transferred into a freshly prepared 15 mL tube placed in ice; 0.2-0.3 V of cold absolute ethanol were added and the tube was immediately shaked vigorously for 1 min.

NOTE AND WARNING: Remove the aqueous phase carefully; avoid jerky movements to prevent re-mixing with interphase layer. Vortex the 15 mL tube immediately following addition of ethanol, to prevent nucleic acid precipitation. Adding ethanol causes precipitation of polysaccharides (Fang et al., 1992).

(20) 2 mL (~1V) of chloroform were immediately added and vortexed vigorously for 1 min.

(21) Using a benchtop shaker, the 15mL tube was mixed for 20 min at 4°C. During the shaking, samples were vortexed every 5-7 min.

(22) The tubes were incubated upright in ice for 20 min.

6.3.3.3. Nucleic acids precipitation (Timing: 2 hours)

(23) Samples were centrifuged at 11,000 rpm for 20 min at 4°C.

(24) The recovered aqueous phase was distributed in 2mL conical tubes as described in Figure 2. To each tube the precipitation mix solutions was added in the indicated order (Table 4). Tubes were inverted 5-10 times.

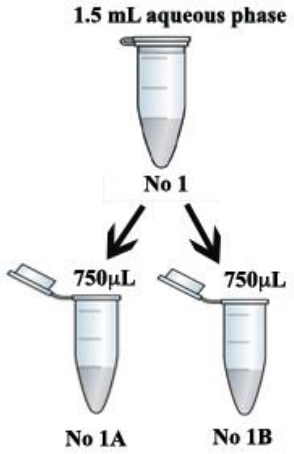

Figure 2. The aqueous phase obtained in step 23 was at least 1.5 mL. By using 2 mL conical tubes it was possible to split and precipitate the aqueous phase of one sample into multiple tubes (usually two), and in a second step join the precipitated nucleic acids.

Table 4. Reagent used in the precipitation step

Aqueous Phase (top layer)		e.g. 1.5 mL		e.g. 1.4 mL	
Aqueous phase split into two tubes		750 µL	750 µL	700 µL	700 µL
Precipitation mix	(0.8 V) Isopropanol	600 µL	600 µL	560 µL	560 µL
	(0.1 V) 3 M sodium acetate, (pH 5.2)	75 µL	75 µL	70 µL	70 µL
	(1%) 2-mercaptoethanol	7.5 µL	7.5 µL	7 µL	7 µL

(25) The nucleic acids were precipitated at -80°C for 1 h, or alternatively at -20°C overnight.

(26) Tubes were centrifuged for 30 min at 13,000 rpm at 4°C to completely precipitate nucleic acids.

NOTE: Do not be alarmed if the samples are frozen after precipitation at -80°C, they will thaw rapidly during centrifugation.

(27) After centrifugation, supernatant was discarded by inverting the tubes over a suitable container; if preferred, a pipette can be used to remove supernatant. Be careful not to dislodge pellet.

NOTE: Since the nucleic acids are completely precipitated at this stage the possibility of cross-contamination is minimal.

2.3.4. Washing DNA/RNA (Timing: 1.5 hours)

(28) The nucleic acid pellet was washed twice with 1mL of cold 75% ethanol to remove contaminants and any residual of 2-mercaptoethanol; centrifuged at 13,000 rpm at 4°C for 20 min.

(29) Any remaining traces of ethanol were removed by a pulse of centrifuge and remainders were collected using a pipette (or by inverting the racked collection of tubes onto absorbent paper), and the pellet allowed to air-dry at room temperature under a laminar flow hood.

NOTE: The nucleic acid pellet is considered dry when no further drops or liquid is observable; it typically occurs within 30-45 minutes. Be careful not to lose the pellet which is securely attached to the tube since rough handling can dislodge it. Moreover, if the pellet is dried upside down for too long, it will fall out.

2.3.5. Dissolving DNA/RNA (Timing: 20-40 minutes)

(30) The pellet with nuclease-free water was hydrated (starting with 25-100 mg biomass the final volume was between 40-50 μL); re-suspension on ice was allowed by gently shaking tubes. The samples were stored at -20°C in the short-term but should be stored at -80°C for longer periods.

NOTE: In step 24, due to the high volume, the supernatant of one sample (e.g. Sample 1) was split and precipitated in two eppendorf tubes (e.g. Samples 1A and 1B) (Figure 2). In this step it is possible to combine the nucleic acids from the two tubes (e.g. Samples 1A and 1B) into one tube by simply join the re-suspended nucleic acids (e.g. Sample 1) as illustrated in Figure 3.

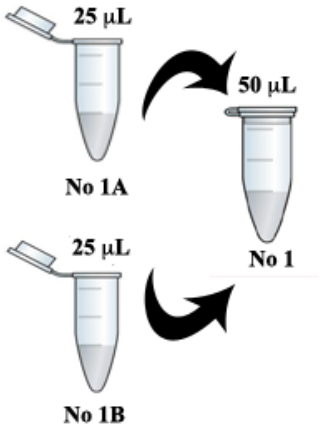

Figure 3. After re-suspension in an appropriate volume of nuclease-free water, the nucleic acids precipitated in two different tubes (step 24) were transferred into a new tube, to obtain a final volume of 40-50 µL.

At this stage co-isolation of DNA and RNA was performed. To obtain pure DNA-free RNA, suitable for sensitive downstream applications such as qRT-PCR, aliquots of nucleic acids were treated with DNase enzyme in order to eliminate genomic DNA contamination.

Conversely, to obtain pure RNA-free DNA, aliquots of the nucleic acid mixture were treated with RNase enzyme.

NOTE: By using primers that bridge exons, mixtures of nucleic acids can be used immediately for reverse transcription and qRT-PCR without DNase treatment (Czechowski et al., 2005). Similarly, since RNA has a very short half-life once extracted and does not impact DNA downstream processes; these can be performed without RNA digestion.

2.3. Purification step

2.3.1.a RNase treatment

(31) To obtain pure DNA, aliquots of nucleic acids mixture were treated with RNase A, DNase free enzyme (Roche Diagnostic Mannheim, Germany) in a final volume of 100 µL, for 20 min at 37°C.

The following mix was added in the indicated order (Table 5). For multiple samples, a master mix can be prepared.

Table 5. RNase treatment of genomic DNA

Component	**Amount**
Genomic DNA	10-25 μg
RNase A (0.1 mg ml^{-1})	1 μL
Nuclease free water	up to 100 μL

2.3.1.b DNase treatment

(31) To obtain pure RNA, aliquots of nucleic acid mixtures were treated with DNase I recombinant, RNase free enzyme (Roche Diagnostic Mannheim, Germany) in a final volume of 50 μL and incubated at 37°C for 17 min.

The following mix was added in the indicated order (Table 6). For multiple samples, a master mix can be prepared.

Table 6. DNase treatment of total RNA

Component	**Amount**
Total RNA	10-25 μg
10X Incubation Buffer	5 μL
10U/μL DNase I recombinant, RNase free enzyme	1 μL
Optionally: Protector RNase Inhibitor (10U)	
Water, RNase free	up to 50 μL

2.3.2. Purification of extracted DNA/RNA (Timing: 1 h).

When purifying nucleic acids it is important to use a method that maintains DNA/RNA integrity whilst removing contaminants. DNA or RNA was purified according the following procedure:

(32) Nuclease-free water was added to the nucleic acids to reach a final volume of 500 μL;

(33) 0.5V of phenol were added and vortexed vigorously for 1 min;

(34) 1 volume of chloroform:isoamyl alcohol was added (24:1, v/v), and vortexed vigorously for 1 min;

(35) The samples were transferred to the thermomixer, and the samples shaked at a mixing speed of 1,300 rpm for 30 min at 10°C; The samples were vortexed every 5 min.

(36) Samples were centrifuged at 13,000 rpm for 25 min at 4°C;

(37) The upper phase was carefully collected (avoiding mixing with interphase layer) and steps 33 to 36 were repeated if the interphase layer showed the presence of proteins/metabolites.

(38) After centrifugation, the upper phase were transferred into freshly prepared 1.5 mL tubes. The precipitation mix solutions were added in the indicated order (Table 7).

Table 7. Precipitation mix composition

Aqueous Phase (top layer)		e.g. 500 µL
Precipitation mix	(0.8 V) isopropanol	400 µL
	(0.1 V) 3 M sodium acetate, (pH 5.2)	50 µL
	(1%) 2-mercaptoethanol	5 µL

(39) Tubes were inverted to mix and incubate at -80ºC for 1 h or at -20°C overnight;

(40) Samples were centrifuged at 13,000 rpm for 30 min at 4ºC. DNA/RNA pellets were washed with 1 mL of cold 75% ethanol, dried and re-suspended in 40 µL of water.

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