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Ecophysiological and metabolic responses to interactive exposure to nutrients and copper excess in the brown macroalga *Cystoseira tamariscifolia*

Paula S.M. Celis-Plá[a,b,*], Murray T. Brown[c], Alex Santillán-Sarmiento[c], Nathalie Korbee[b], Claudio A. Sáez[a], Félix L. Figueroa[b]

[a] Laboratory of Coastal Environmental Research, Center of Advanced Studies, University of Playa Ancha, Traslaviña 450, 581782 Viña del Mar, Chile
[b] Department of Ecology and Geology, Faculty of Sciences, University of Malaga, 29071 Malaga, Spain
[c] School of Marine Science and Engineering, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK

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ABSTRACT

Global scenarios evidence that contamination due to anthropogenic activities occur at different spatial-temporal scales, being important stressors: eutrophication, due to increased nutrient inputs; and metal pollution, mostly derived from industrial activities. In this study, we investigated ecophysiological and metabolic responses to copper and nutrient excess in the brown macroalga *Cystoseira tamariscifolia*. Whole plants were incubated in an indoor system under control conditions, two levels of nominal copper (0.5 and 2.0 μM), and two levels of nutrient supply for two weeks. Maximal quantum yield (F_v/F_m) and maximal electron transport rate (ETR_{max}) increased under copper exposure. Photosynthetic pigments and phenolic compounds (PC) increased under the highest copper levels. The intra-cellular copper content increased under high copper exposure in both nutrient conditions. *C. tamariscifolia* from the Atlantic displayed efficient metal exclusion mechanisms, since most of the total copper accumulated by the cell was bound to the cell wall.

1. Introduction

Marine biota living in coastal waters are under constant threat from exposure to elevated concentrations of pollutants, such as metals and nutrients, mostly derived from domestic, industrial and farming activities (Ferreira et al., 2011). In near-shore ecosystems, macroalgae are the dominant primary producers; within the latter, brown seaweeds (Phaeophyceae) are particularly important bio-engineer organisms (Litter and Litter, 1984; Wells et al., 2007; Celis-Plá et al., 2016, 2017), providing shelter, food and habitat for many other marine biota (Graham et al., 2007; Sáez et al., 2012).

Stress biology research on metal (and particularly copper)-stressed brown seaweeds has shown different levels of physiological, biochemical and molecular detrimental effects, as it has been observed in *Ascophyllum nodosum* (*e.g.* Connan and Stengel, 2011a, 2011b), *Fucus vesiculosus* (*e.g.* Nielsen and Nielsen, 2010) and *Ectocarpus siliculosus* (*e.g.* Roncarati et al., 2015; Sáez et al., 2015a). Even though copper is an essential metal at trace levels, for instance as co-factor in several enzyme complexes, beyond certain threshold concentrations it can become toxic and affect metabolic and physiological performance (Connan and Stengel, 2011a, 2011b; Roncarati et al., 2015; Moenne et al., 2016). Copper excess can have negative effects on the metabolism of macroalgae through different known pathways (Sáez et al., 2015a). This involve the induction an oxidative stress condition and the substitution of other essential metals in biomolecules. In the case of the copper, this can replace magnesium in the chlorophyll molecule, incapacitating it to perform photosynthesis (Küpper et al., 2002; Moenne et al., 2016). In *A. nodosum* and *F. vesiculosus*, the ecophysiological responses were in detrimental under increase copper (1.6 μM for 15 d), causing an inhibition in photosynthesis and degradation of seaweed tips (Connan and Stengel, 2011a, 2011b). In terms of metabolic responses, the copper at 2.4 μM for 7 d in the brown macroalga *E. siliculosus* showed increased levels of lipid peroxidation and H_2O_2 content with respect to without copper conditions, and displayed signs of oxidative stress and damage (Sáez et al., 2015a). Furthermore, *E. siliculosus* under increase copper at 2.4 μM increased antioxidant defences by means of increased content of phenolic compounds and greater production and activities of antioxidants and antioxidant enzymes, respectively, associated with the glutathione-ascorbate cycle were detected (Sáez et al., 2015a).

It is known that nitrate and phosphate represent important macronutrients for macroalgae development and in addition can protect the algae against stress. For instance, high concentrations of nutrients in seaweeds can reduce photoinhibition, as it has been observed in

* Corresponding author at: Laboratory of Coastal Environmental Research, Center of Advanced Studies, University of Playa Ancha, Traslaviña 450, 581782 Viña del Mar, Chile.
E-mail address: paulacelispla@upla.cl (P.S.M. Celis-Plá).

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Cystoseira tamariscifolia under 50 μM nitrate (Celis-Plá et al., 2014a) and *Ulva lactuca* subject to 239 μM nitrate (Figueroa et al., 2009). Other observations showed that nutrient enrichment could also have positive effects on photosynthesis, photo-protection and biochemical responses (Celis-Plá et al., 2016). Indeed, *Cystoseira tamariscifolia* from Southern Mediterranean Sea showed that photosynthetic performance and the concentration of phenolic compounds were higher under 50 μM nitrate (Celis-Plá et al., 2014a). In contrast, individuals of *C. tamariscifolia* from ultraoligotrophic waters (Cabo de Gata-Nijar Natural Park) showed greater photoinhibition and ecophysiological performance under 107 μM nitrate and 24 μM phosphate contents (Celis-Plá et al., 2014b). Certainly, the available information on the combined effects of metal-excess and increased nutrients is scarce in macroalgae; according to research available published, *e.g.*, Huovinen et al., 2010. This study showed the most copper accumulation in *Macrocystis*, which decreased under nitrate-enriched conditions, as well as, the inhibition of photosynthetic activity by copper. Thus, the investigation on the combined effects of nutrients and metals excess studying in brown seaweeds would provide relevant information about their capacity to withstand the future pollution scenarios. The interaction between metals and nutrients excess is still not well understood for macroalgae. In this study, we analyse the physiological and biochemical responses under different copper and nutrient levels, using standard methods for the study of multiple physical stressors in algae (Martínez et al., 2012; Celis-Plá et al., 2014b). We investigate the interactive effects of excess copper and macronutrients (phosphate and nitrate) on certain parameters associated with physiological and metabolic responses in the brown seaweed *C. tamariscifolia*. Nitrogen and carbon internal content, photosynthetic pigments (chlorophylls and fucoxanthin), intracellular and released phenolic compounds, phenolic content, antioxidant capacity, and total and intra-cellular copper content, were measured. Additionally, photosynthetic activity was assessed by comparing parameters derived from measurements by using *in vivo* chlorophyll *a* fluorescence.

2. Material and methods

2.1. Species, sampling and experimental design

Whole thalli of *Cystoseira tamariscifolia* (Hudson) Papenfuss, (Phaeophyceae, Fucales) (Gómez-Garreta et al., 2001; Bunker et al., 2010) were collected randomly on 6 May 2014 in Hannafore Point, Cornwall (50°36′N, 4°42′W), Atlantic Ocean. Seawater from this site have been described to have nitrate concentrations of around 5.0 μM (Woodward et al., 2013).

C. tamariscifolia (approximately 2 or 3 plants; in total 30 g per open tank of the fresh weight of individuals) were incubated for 14 days (*s*), from 8 to 22 of May 2014 (after 48 h of acclimation). The algal material was previously cleaned out of epiphytes manually under running seawater. The experiment was designed to examine interactive effects of copper (as $CuSO_4{\cdot}5H_2O$), at control copper (seawater with no copper added), at 0.5 μM (low copper levels) and 2.0 μM (high copper levels), and nutrient conditions, at control or natural seawater, and at 50 μM KNO_3 plus 5 μM KH_2PO_4 (nutrient enrichment). The six treatments were: control copper and natural seawater (CCNS); control copper and nutrient enrichment (CCNP +); low copper and natural seawater (LCNS); low copper and nutrient enrichment (LCNP +); high copper and natural seawater (HCNS); and high copper and nutrient enrichment (HCNP +). In total, 18 open tanks of methacrylate were used, with three replicates per treatment.

2.2. Experimental conditions

The experimental system consisted in 18 open tanks (0.030 m^2 surface area, 3.0 L volume), with seawater continuously aerated. Water temperature was monitored using a HOBO logger (Onset Computer Corporation, Massachusetts, USA). The photosynthetically active radiation PAR (λ = 400–700 nm) was provided using cool white fluorescent lamps (Osram FH 21W/840HE, Luminox, Italy), and with a 14:10 h light/dark cycle. Seawater was changed every two days.

2.3. Physiological and biochemical variables

Several physiological variables were measured in the algae of each open tank after one week (7 days) and the end of the experiment (14 days). Nitrogen and carbon contents were determined in fronds using an element analyzer CNHS-932 model (LECO Corporation, Michigan, USA) (according to Celis-Plá et al., 2016). Nitrogen and carbon were expressed as mg g^{-1} dry weight (DW) after determining fresh weight (FW) to DW ratio in the tissue (8.17 for *C. tamariscifolia*).

2.4. Photosynthetic activity

In vivo chlorophyll *a* fluorescence associated with photosystem II was determined using a portable pulse amplitude modulated fluorometer Diving-PAM with a WinControl Software V3.25 (Walz GmbH, Germany). Pieces of the apical parts (one piece for replicate) of the fronds of *C. tamariscifolia* were collected at 7 days (middle time) and after 14 days (for each tank) and they were placed in the 10 mL incubation chambers in order to conduct rapid light curve, one for each tank. F_o (basal fluorescence yield) and F_m (maximum fluorescence yield) were determined after 15 min in darkness to obtain the maximum quantum efficiency of PSII (F_v/F_m), were $F_v = F_m - F_o$, F_o is the basal fluorescence of dark-adapted thalli after 15 min and F_m is the maximal fluorescence after a saturation light pulse of > 4000 μmol m^{-2} s^{-1}) (Schreiber et al., 1995). Electron transport rates (ETR, μmol electrons m^{-2} s^{-1}) as rapid light curve (RLC) was determined after 20 s exposure period in 12 increasing irradiance (9.3, 33.8, 76, 145, 217, 301, 452, 629, 947, 1403, 2084 and 3444 μmol m^{-2} s^{-1}) of white light, (halogen lamp of the Diving-PAM). ETR was calculated according to Schreiber et al. (1995) as follows:

$$\text{ETR}\ (\mu\text{mol electrons}\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (1)$$

where $\Delta F/F'_m$ is the effective quantum yield, $\Delta F = (F'_m - Ft)$, (Ft is the intrinsic fluorescence of alga incubated in light and F'_m is the maximal fluorescence reached after a saturation pulse of the algae incubated in light). E is the incident PAR irradiance expressed in μmol photons m^{-2} s^{-1}, A is the thallus absorptance as a fraction of incident irradiance that is absorbed by the alga (Figueroa et al., 2003), and F_{II} is the fraction of chlorophyll related to PSII (400–700 nm), being 0.8 in brown macroalgae (Grzymski et al., 1997). Maximum ETR (ETR_{max}, estimate of maximal photosynthetic capacity), and the photosynthetic efficiency (α_{ETR}) the initial slope of the ETR curve (estimate of photosynthetic efficiency) were obtained from the tangential function reported by Eilers and Peeters (1988).

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$\text{NPQ} = (F_m - F'_m)/F' \quad (2)$$

Maximal non-photochemical quenching (NPQ_{max}) is considerate as indicator of energy dissipation and as photoprotection mechanisms (Celis-Plá et al., 2016). NPQ_{max} was obtained from the tangential function of NPQ *versus* irradiance according to Eilers and Peeters (1988).

2.5. Pigment content

Pigments were extracted from 20 mg FW of fronds using 800 μL of dimethyl sulfoxide (DMSO) and 200 mL. After 5 min, samples were diluted with distilled water in a ratio of 4:1 (DMSO: water), and the absorbance (A) was determined at a spectrophotometer (Jenway 7315, Cole-Parmer, UK) at specific wavelengths (subscripts in equations