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Differential responses of *Emiliania huxleyi* (Haptophyta) strains to copper excess

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Résumé – *Emiliania huxleyi* est un coccolithophore cosmopolite qui présente un haut degré de variabilité génétique et physiologique aussi bien entre souches qu'entre les étapes de son cycle de vie. Ici, nous avons examiné si les réponses au cuivre (Cu) et au stress oxydatif varient selon l'origine environnementale ou l'étape du cycle de vie. Nous avons comparé les réponses à des concentrations toxiques de Cu et à des expositions à court terme au peroxyde d'hydrogène de douze souches (huit diploïdes et deux paires de souches où haploïdes and diploïdes partagent la même base génétique) provenant de différentes origines océanographiques. Les réponses mesurées comprenaient le taux de croissance, les proportions de coccolithes malformés/incomplètes (souches diploïdes), la motilité (souches haploïdes), la fluorescence variable (Fv/Fm, une mesure de la santé de photosystème), analyses de cytométrie en flux des pourcentages de cellules vivantes et l'accumulation des dérivés réactives de l'oxygène (DRO), et les activités des enzymes ascorbate peroxydase (AP) et glutathion peroxydase (GP). Bien qu'une importante variabilité ait été observable entre les réponses au stress de ces souches, aucune différence statistique n'a été détectée lorsque les souches ont été regroupées par origines. Dans les deux paires diploïdes/haploïdes, les diploïdes ont montré une plus grande sensibilité à l'inhibition de la croissance par Cu que leurs haploïdes correspondants, bien que cette différence n'était significatif que dans le cas de la paire diploïde-haploïde qui est issue d'un isolât de la mer de Tasman (RCC1216 / RCC1217). Les diploïdes accumulent rapidement DRO en réponse à une exposition aiguë à Cu, alors que l'accumulation de DRO dans haploïdes était beaucoup plus faible. Notablement,

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il n'y a eu pas des différences dans les DRO induites par Cu entre les diploïdes sensibles et résistantes, ou entre les haploïdes sensibles et résistantes. Les différences de sensibilité au Cu entre les souches ont été reflétées dans les différences d'activité AP constitutive, mais aucune différence entre les souches dans l'activité GP constitutive ou induite n'a été détectée. Les différences dans l'activité AP constitutive, mais non dans l'activité GP induite par Cu, reflètent la sensibilité au Cu. Dans l'ensemble, nos résultats montrent une variabilité intra-spécifique élevée parmi les génotypes et les phases du cycle de vie de *E. huxleyi*, ce qui pourrait refléter en partie les différences dans la tolérance au stress oxydatif.

Stress par cuivre / coccolithophores / *Emiliania huxleyi* / réponse souche-dépendant / cycle de vie

Abstract – *Emiliania huxleyi* is a cosmopolitan coccolithophore exhibiting a high degree of genetic and physiological variability among strains and life-cycle stages. Here we investigated whether responses to copper (Cu) toxicity and oxidative stress varied by environmental origin or life cycle stage. We compared responses to toxic concentrations of Cu and short-term exposure to hydrogen peroxide of twelve strains (eight diploids and two pairs of strains where haploids and diploids shared a genetic background) from different oceanographic origins. Measured responses included growth rate, proportions of malformed/incomplete coccoliths (diploid strains), motility (haploid strains), variable fluorescence (F_v/F_m, a measure of photosystem health), flow cytometry assays of the percentages of live cells and the accumulation of intracellular reactive oxygen (ROS), and the activities of ascorbate peroxidase (AP) and glutathione peroxidase (GP). Although a large and significant variability was observable in Cu sensitivity among strains, no statistical differences were detected when strains were grouped by their origins. In the two diploid/haploid pairs, the diploids showed more sensitivity to growth inhibition by Cu than their corresponding haploids, although this was only large and significant in the case of diploid-haploid pair that originated from a Tasman Sea isolate (RCC1216/RCC1217). The diploids accumulate ROS rapidly in response to acute exposure to Cu, while ROS accumulation in haploids was much lower. Notably, there was no difference in Cu-induced ROS between the sensitive and resistant diploids or between the sensitive and resistant haploids. Differences in Cu-sensitivity between strains were reflected in differences in constitutive AP activity, but no differences between strains in constitutive or induced GP activity were detected**.** Overall, our results show a high intra-specific variability among genotypes and life-cycle phases in *E. huxleyi*, which might partly reflect differences in tolerance to oxidative stress.

Copper stress / coccolithophores / *Emiliania huxleyi* / strain-specific responses / life cycle

INTRODUCTION

Emiliania huxleyi (Lohmann) Hay et Mohler, is one of the most widely distributed microalgae as this morph-species is found in most marine surface waters outside the polar oceans (Brown & Yoder, 1994; Winter *et al.*, 1994). This coccolithophore, capable of forming dense and extensive blooms in many temperate systems (Paasche, 2002; Moore *et al.*, 2012), plays an important role in carbon and sulfur biogeochemical cycles through calcification and dimethylsulfoniopropionate (DMSP) production (Keller 1989; Holligan *et al.*, 1993; Westbroek *et al.*, 1993; Paasche 2002).

Strains of *Emiliania huxleyi* exhibit a high degree of intraspecific phenotypic, genetic, and genomic variability. Physiological variability has been demonstrated in traits or responses including growth rates (Brand, 1982), temperature-dependence of growth rates (Brand, 1982; Zhang *et al.*, 2014), calcification (Young & Westbroek, 1991; Cubillos *et al.,* 2007), susceptibility to viruses (Bidle *et al.*, 2007), responses to nutrient limitation (Oviedo *et al.,* 2014), responses to ocean acidification (Langer *et al.*, 2009; Meyer & Riebesell, 2015), and in particulate organic and inorganic carbon contents, C:N, Mg:Ca and Sr:Ca ratios, (Blanco-Ameijeiras *et al.*, 2016). This phenotypic variability reflects high genetic (Medlin *et al.*, 1996; Iglesias-Rodriguez *et al.*, 2006) and genomic variability (Kegel *et al.*, 2013; Read *et al.*, 2013; von Dassow *et al.*, 2015). Most of these studies in *E. huxleyi* have focused on a few strains and only the diploid stage. However, this species alternates between the calcified non-motile diploid stage and a motile haploid stage that is not calcified, with both stages potentially capable of unlimited asexual reproduction (Klaveness, 1972; Green *et al.*, 1996; von Dassow *et al.*, 2009). Nevertheless, the works which have compared both stages have pointed out important ecophysiological and transcriptomic differences (von Dassow *et al.*, 2009; Rokitta *et al.*, 2011), including responses to light acclimation (Houdan *et al.*, 2005; Rokitta *et al.*, 2011; Rokitta & Rost, 2012), nitrate and phosphate starvation (Rokitta *et al.*, 2014, Rokitta *et al.*, 2016), elevated pCO_2 (Rokitta *et al.*, 2012; Rokitta & Rost, 2012), and viruses (Frada *et al.*, 2008). Furthermore, a recent work has revealed that many *E. huxleyi* strains have lost essential genes required in the haploid phase for formation of flagella (von Dassow *et al.*, 2015). Interestingly, the tendency to lose the capacity to form flagellated cells is almost exclusively observed in strains originating from open ocean conditions, whereas strains isolated from coastal environments tend to maintain the capacity to complete the full life cycle. It has been suggested that different adaptive strategies have permitted this single morpho-species to colonize distinct ocean environments (Read *et al.*, 2013).

Among the environmental factors that vary the most in the ocean and that might drive local adaptations are micronutrients such as metals. Pronounced differences between the trace metal requirements of coastal and oceanic diatoms isolates of the same genera have been shown, emphasizing a significant habitat effect (Maldonado & Price, 1996; Annett *et al.*, 2008; Guo *et al.*, 2010). Metals such as copper (Cu) are particularly intriguing because they are required for growth at low concentrations but become toxic at high concentrations (Pinto *et al.*, 2003). The high variability of Cu between open ocean and coastal waters is due mainly to the anthropogenic input from land and rivers in the latter (Moffett, 1995; Lee & Correa, 2005; Andrade *et al.*, 2006; Nogales *et al.*, 2011). Aeolian input of Cu can also be important, and has been implicated as a factor in repressing phytoplankton growth at large scales (Jordi *et al.*, 2012). Those authors showed that the input of Cu from aerosols was related to negative changes in satellite chlorophyll-a (Chl-a) in the Mediterranean and proposed that similar processes could even occur in major regions of the Indian, Atlantic, and Pacific oceans. Meanwhile, in the pico-cyanobacteria *Synechoccus*, coastal strains have been found to have genomic islands conferring higher Cu resistance compared to oceanic strains (Stuart *et al.*, 2013), supporting the idea that tolerance to metal-toxicity may be a trait that can be subject to strong selection in marine phytoplankton.

In phytoplankton, Cu plays an important role as cofactor in a number of enzymes, such plastocyanin, cytochrome c oxidase, Cu/Zn superoxide dismutase (SOD) and some high-affinity Fe transport systems (Raven *et al.*, 1999; Maldonado *et al.*, 2006; Peers & Price, 2006). However, Cu is toxic at high levels due to its

high reactivity and ability to disturb redox balance leading to oxidative stress, characterized by increased production of reactive oxygen species (ROS) (Erickson, 1972; Harrison *et al.*, 2007; Knauert & Knauer, 2008). The ROS over-production under Cu excess is a similar response to other stressor conditions, such as viral infection (Evans *et al.*, 2006), nutrient starvation (Allen *et al.*, 2008), desiccation (Contreras-Porcia *et al.*, 2011), and UV and high light irradiation (Mella-Flores *et al.*, 2012). Either directly or through ROS, Cu can induce effects on various biochemical and physiological processes, such as inhibition of cell division (Prasad *et al.*, 1998; Lage *et al.*, 2001), photosynthesis (Perales-Vela *et al.*, 2007), respiration (Xia & Tian, 2009), synthesis of pigments (Rijstenbil *et al.*, 1994), and cell motility (Lage *et al.*, 2001). Several organisms have developed antioxidant responses to cope with Cu stress, including the use of antioxidant enzymes (ascorbate peroxidase, glutathione peroxidase, catalase, among others), water-soluble antioxidant compounds, and lipid-soluble antioxidant molecules (Foyer *et al.*, 1997; Noctor & Foyer, 1998). Thereby, sensitivity and survival capacity will depend upon the amount of ROS produced and their efficiency in scavenging these oxygen species.

The sensitivity to Cu is highly variable among microalgae. It is often determined by measuring the effective Cu concentration that inhibits 50% microalgal growth (IC_{50}), and by this measure cyanobacteria and diatoms are reported to be especially sensitive (<1 μg Cu L^{-1}) (Brand *et al.*, 1986; Sunda & Huntsman, 1995; Levy *et al.*, 2007). On the other hand, the Chlorophyta *Dunaliella tertiolecta* and *Parachlorella kessleri* are very resistant (>500 μg Cu L^{-1}) (Levy *et al.*, 2007; Nugroho & Frank, 2011). Tolerance ranges may be due to morphological and structural factors such as size, cell volume, and presence/absence of a cell wall or mucilaginous sheets, as well as physiological or biochemical mechanisms for metal tolerance or ability to deal with elevated ROS induced by metals (Levy *et al.*, 2007). In this context, *E. huxleyi* exhibits a moderately high tolerance to Cu (>10 μg Cu L^{-1}) (Brand *et al.*, 1986; Levy *et al.*, 2007).

The aim of this work was to test if strains of *Emiliania huxleyi* isolated from distinct ocean environments may display different degrees of responses to Cu stress and whether responses depend on life cycle stage. Furthermore, we evaluated if these responses are correlated to stress induced by hydrogen peroxide, as a universal and direct oxidative stressor. For that, different aspects of Cu toxicity were evaluated, such as IC_{50} for inhibition of growth, photosynthetic efficiency (F_v/F_m), intracellular ROS accumulation, motility, formation of coccoliths, and antioxidant activities of the enzymes ascorbate peroxidase and glutathione peroxidase involved in ROS scavenging (Lesser, 2006).

MATERIAL AND METHODS

Strains

Twelve strains of *Emiliania huxleyi* were used in this study, among which ten were diploid and two were haploids (Table 1). Briefly, six strains were isolated from South East Pacific waters (three from coastal waters and three from oceanic waters) and four were obtained from the Roscoff Culture Collection (RCC) with origins in the Tasman Sea and the Mediterranean Sea. From the RCC strains, the two Tasman Sea strains originate from the same genetic background, that is, a clonal

Table 1. *Emiliania huxleyi* strains used in this study: name, origin and morphotype

Strain name	*Geographical origin*	*Coordinates*	*Oceanographic regime*	*Date of original isolation*	*Morphotype*
RCC1216	Tasman Sea	42º18'00.0"S. 169º50'00.0"E	Transitional	01.09.1998	R
RCC1217[a]	Tasman Sea	42º18'00.0"S. 169º50'00.0"E	Transitional	01.09.1998	Motile form
F2[b]	Mediterranean Sea	34º08'00.0"N. 18º27'00.0"E	Oligotrophic	01.09.2008	B
B4[b]	Mediterranean Sea	34º08'00.0"N. 18º27'00.0"E	Oligotrophic	01.09.2008	Motile form
CHC350[c]	Chilean Coast	30°14'49.0"S. 71°41'38.0"W	Coastal upwelling	19.11.2012	A – overcalcified
CHC352	Chilean Coast	30°14'49.0"S. 71°41'38.0"W	Coastal upwelling	19.11.2012	R
CHC366[c]	Chilean Coast	30°14'49.0"S. 71°41'38.0"W	Coastal upwelling	19.11.2012	A – overcalcified
CHC428[c]	South Pacific	16º44'56.4"S. 85º59'52.8''W	Oceanic	09.07.2013	A
CHC440	South Pacific	16º44'56.4"S. 85º59'52.8''W	Oceanic	09.07.2013	A
CHC524	South Pacific	16º44'56.4"S. 85º59'52.8''W	Oceanic	09.07.2013	A
CHC299[c]	Juan Fernández Islands	33º39'03.7"S. 78º36'08.0''W	Oceanic	01.11.2011	A
CHC307[c]	Juan Fernandez Islands	33º39'03.7''S. 78º36'08.0''W	Oceanic	01.11.2011	A

[a] 1N strain originated from the 2N RCC1216 cell. one year after isolation of RCC1216
[b] B4 is a 1N strain isolated from RCC1855 on 15.03.2010. which was isolated as a calcified cell on 01.09.2008. F2 is a 2N strain that originated in culture from the 1N B4 strain on 02.06.2010.
Morphotype descriptions from Hagino et al. 2011
[c] CHC350, CHC366, CHC428, CHC299, and CHC307 are also available as RCC4028, RCC4029, RCC4030, RCC3973, RCC4002 (respectively) in the Roscoff Culture Collection.

diploid (2N) strain (RCC1216) that formed haploid (1N) cells in culture (RCC1217). Similarly, Mediterranean strains B4 and F2 are derived strains isolated from the original RCC1855 strain when spontaneously flagellated cells appeared in the culture (B4). Then, from B4, new calcified cells appeared and were isolated giving origin to F2. All strains are currently kept in the South Eastern Pacific Algae Collection, Pontificia Universidad Católica de Chile.

Culture conditions

Cultures were grown at 14°C ± 1°C in a seawater-based medium enriched with 176 μM nitrate, 8 μM phosphate, trace metals at half the concentrations of K/2 medium (Keller & Guillard 1985; Keller *et al.*, 1987), and full-strength K/2 vitamins. Seawater was collected from the central Chilean coast during winter, aged for several months, and sterile-filtered at 0.22 μm (Merk Millipore Corporation, Billerica, MA) before being used in the experiments. All experiments were performed under cool white fluorescent light illumination with a photoperiod of 12:12 light-dark cycle and irradiance of 45-50 μmol photons m^{-2} s^{-1}.

Copper toxicity bioassays

The toxicity of Cu to 12 *Emiliania huxleyi* strains was determined using growth-rate inhibition bioassays. Composition of the medium was as described above with some further modifications: seawater was treated with active carbon to eliminate dissolved organic matter in the water, trace metals were diluted at 1/50 the concentration of K/2 and no Cu was added. Preliminary experiments confirmed that cultures showed no difference in growth between media with the standard or the lower trace metal concentrations, at least at cell densities up to several hundred cells mL^{-1}, much higher than the maximum cell densities reached in these experiments. Furthermore, the EDTA concentration was decreased further in the assays (10 μM, final concentration in medium) in order to diminish the chelating effect of EDTA on copper. Trace metal clean techniques were used throughout the whole experiment period, following EPA protocols (EPA, 1996). All the glass and plastic materials used were soaked overnight in 5% Extran detergent (Merk Millipore Corporation, Billerica, MA) to remove organic matter, washed with distilled water, and soaked in 10% HCl for at least 24 h before being rinsed abundantly with Milli-Q water and stored in acid-cleaned plastic bags until use. Bioassays were carried out in 100 mL borosilicate Erlenmeyer flasks containing 50 mL of medium with initial cell density of 3.500 cells mL^{-1}. Inoculations were provided from a culture in exponential growth phase after three washes of cells by centrifugation (500 × *g* for 10 min the first wash and 200 × *g* for 10 min the following) in a centrifuge with a swinging bucket rotor (Model 5702, Eppendorf, Hamburg, Germany) and with re-suspension in sterile medium without copper. Cells were incubated for 96 h (and until 144 h for the strains with slower growth rates) with seven increasing nominal Cu concentrations (0, 1.5, 4, 10, 20, 45 and 100 μg Cu L^{-1}) prepared from cupric chloride standard solution (Merk Millipore Corporation, Billerica, MA). These concentrations were selected based on a wide-range preliminary test (data not shown) and on nominal concentrations reported to inhibit growth in *E. huxleyi* and other phytoplankton groups (Brand *et al.*, 1986; Levy *et al.*, 2007; Perales-Vela *et al.*, 2007; Manimaran *et al.*, 2012). During growth tests, flasks were rotated within the culture chamber and gently mixed every day in order to ensure sufficient gas exchange and reduce

cell sedimentation. Growth rate was monitored daily by fluorometric measurements of *in vivo* chlorophyll *a* fluorescence using a handheld fluorometer (AquaFluor, Turner Designs, San Jose, CA, USA). Direct cells counting were also performed using an improved Neubauer chamber to confirm trends observed by chlorophyll fluorescence (data not shown). Samples were taken at the end of the essays for Scanning Electronic Microscopy (SEM) observations, photosynthesis activity measurements, and motility determination. All bioassays were performed in triplicates.

Growth parameters and IC_{50} calculations

The growth rate (μ) was calculated during exponential phase by fitting a linear regression curve to the logarithmic transformed cell fluorescence over time: $F=F_0 e^{\mu t}$, where, F_0: Fluorescence at time zero and F: Fluorescence at time t (days). The use of cell fluorescence as growth parameter was checked by direct cell counting on a sub-set of experiments, yielding very similar growth rates and nominal IC_{50} values (data not shown). The 96h-IC_{50} values were obtained for each strain by fitting growth inhibition data to a dose-response equation using Prism 6.0h software (GraphPad Software, Inc., San Diego, California, USA). Previously to the fitting, data were first transformed to convert the X values (Cu concentration) to logarithms and then normalized Y values (Chl fluorescence) by defining 0 and 100 % of the response as the minimum and maximum values of each data set.

Because the growth rate of controls presented a wide variation among strains and this might potentially affect IC_{50} calculations for more slowly growing strains, IC_{50} values were also calculated based on the day when controls had completed a minimum of 3 generations (doublings), and exposure time was constrained between a minimum of 96 h and a maximum of 144 h (MinEx-IC_{50}), restrictions determined from preliminary experiments to keep cell densities below 10% of maximum achieved during stationary phase and thus avoid nutrient limitation (not shown).

Effect of copper on coccolith morphology and motility

Morphology of coccoliths was evaluated by SEM observation on the last point of the growth-rate inhibition bioassays in selected diploid strains. One mL of cell suspension of each strain was sampled and fixed by addition of 0.1 volumes of 100 mM sodium borate pH 8.7 solution containing 10% formaldehyde and 0.5% glutaraldehyde (von Dassow *et al.*, 2012) and kept at 4ºC until processing. Samples were then filtered onto polycarbonate Isopore membrane filters (TSTP or GTTP of 1.2 μm, Millipore, MA, USA). Small sections of dried filter samples were mounted on SEM supports, sputter-coated with gold and analyzed under a Hitachi Tabletop Microscope 3000 (Hitachi High-Technologies Corporation, Tokyo, Japan). Percentage of complete and incomplete/malformed coccoliths was calculated by observing a minimum of 49 (F2, 100 μg Cu L^{-1}) and maximum of 416 coccoliths (RCC1216, 0 μg Cu L^{-1}).

Motility was evaluated by light microscopy at the last point of the growth-rate inhibition bioassays for haploid cultures by quantifying the proportion of motile cells in 12-30 random fields at 10X (CKX41 inverted microcope, Olympus, Tokyo, Japan). We note that the movement of cells was not fast enough to escape the observation field during the counting. Only the B4 strain was evaluated since cells

from RCC1217 strain, the other haploid representative, did not show substantial motility in control culture conditions.

As both SEM and motility analyses were highly time intensive, we focused on the 0, 10 and 100 μg Cu L^{-1} treatments, picking the extreme values of the range tested and an intermediate value that was above the IC_{50} for growth inhibition for the most sensitive strains but below the IC_{50} value for the most resistant strains.

Peroxide stress assays

In order to evaluate short-term response to direct oxidative stress, cells from selected strains representative for coastal (CHC350, CHC366) and oceanic conditions (CHC524 and CHC299), and haploid (B4, RCC1217) and diploid life cycle stages (F2, RCC1216) were subjected to H_2O_2 stress by incubation in K/2 medium with three H_2O_2 concentrations (60, 250 and 500 μM). The peroxide concentrations were chosen based on preliminary tests and on concentrations previously reported to affect microalgae (Evans, 2006; Ross *et al.*, 2006; Mella *et al.*, 2012). H_2O_2 bioassays were carried out in 250 mL flasks containing 150 mL of medium with a cell density between 150,000 and 200,000 cells mL^{-1}. After incubation for 30 min in standard culture conditions, the effect of these treatments on the PSII quantum yield (F_v/F_m) was measured as described below.

Variable fluorescence

The variable fluorescence parameter PSII quantum yield (F_v/F_m) was determined using the AquaFluor handheld fluorometer. After 20 min of dark adaptation in controlled culture conditions, an excitation light (460 nm) was applied on a 2 mL aliquot in order to measure the basal fluorescence (F_0). The maximal fluorescence F_m was determined following the addition of 50 μM of the PSII inhibitor 3-(30, 4-dichlorphenyl)-1,1-dimethylurea (DCMU), which blocks electron transport from PSII, following the methodologies used in early studies of variable fluorescence (Samuelsson & Oquist, 1977; Cullen & Renger, 1979; Kromkamp & Forster, 2003). The PSII quantum yield was calculated as: $F_v/F_m = (F_m-F_0)/F_m$.

Cell viability assay

In order to evaluate if responses to Cu exposure were associated to cell death, we evaluated cell viability on three strains, two diploids (F2 and RCC1216) and one haploid (RCC1217) using the fluorescent probe fluorescein diacetate (FDA, ThermoFisher Scientific, San Jose, CA). This probe accumulates in cells as fluorescein if metabolic (esterase) activity is present and the plasma membranes is intact (Selvin *et al.*, 1989; Li *et al.*, 2011). The FDA staining protocol was adapted from Jochem (1999) and Li *et al.* (2011). A stock solution of 5 mg mL^{-1} FDA was prepared in DMSO and a working solution was obtained by diluting the stock solution 20x in Milli-Q water. For the assay, selected strains were submitted to 0 and 100 μg L^{-1} Cu for 24 h in triplicates. After this time, samples were incubated in the dark for 20 min with the working solution in a 4-fold dilution, and FDA staining was measured immediately by flow cytometry. Fluorescence of FDA and chlorophyll were measured, respectively, at 530 nm (40 nm bandpass) and 692 nm (40 nm bandpass) on an InFlux flow cytometer (Becton, Dickinson and Company,

San Jose, CA) with excitation by 488 nm laser. Autofluorescent calibration particles (3 μm UltraRainbow, Spherotech, Lake Forest, IL, USA) were used to align optics and standardize instrument performance. Signals were analyzed with the FlowJo software (Ashland, Oregon, USA). *Emiliania huxleyi* cells were discriminated on the basis of their red autofluorescence (692 nm). A threshold value of green fluorescence for discriminating FDA-positive (live) cells was determined based on the 95th percentile fluorescence value of cells killed by chemical fixation prior to FDA staining.

Detection of antioxidant enzyme activities

To measure the constitutive and Cu-induced activities of the antioxidant enzymes ascorbate peroxidase (AP) and glutathione peroxidase (GP), cultures in triplicate were grown in 500 mL volumes to a cell concentration of 100,000 cells mL^{-1} and exposed to 0 and 100 μg Cu L^{-1}. After 24 h, the totality of the culture was harvested by centrifugation at 1,000 × *g* for 10 min at 4ºC followed by a second centrifugation in microtubes (10,000 × *g*, 1 min at 4ºC). Cells pellets were frozen in liquid nitrogen and kept at -80ºC until analysis. Re-suspended cells were sonicated three times in 2 mL of 0.1 M phosphate buffer pH 7.0 for 10 s in order to disrupt cells. Protein concentration was obtained using the bicinchoninic acid assay (Smith *et al.*, 1985) by measuring directly from the sonicated cells. The ascorbate peroxidase (AP) activity was determined as described previously (Contreras *et al.*, 2005). The reaction mixture contained 0.1 M phosphate buffer pH 7.0, 800 μM ascorbate (ASC) and 16 mM H_2O_2. After the addition of ASC, its consumption was determined at 290 nm for 5 min and the activity was calculated using the extinction coefficient of ASC (ε= 2.8 $mM^{-1}cm^{-1}$). The glutathione peroxidase (GP) activity was measured as described by (Ursini *et al.*, 1985). Briefly, the reaction mixture contained to 0.1 M phosphate buffer, pH 7.0, 200 mM GSH, 8 mM H_2O_2, 90 mM NADPH, and 1 U of glutathion reductase (Sigma). After the addition of NADPH, its oxidation was monitored at 340 nm for 5 min, and GP activity was calculated using the extinction coefficient of NADPH (56.2 $mM^{-1}cm^{-1}$).

Intracellular ROS accumulation

ROS accumulation was evaluated on four strains, two haploids (RCC1217 and B4) and two diploids (RCC1216 and F2) by the fluorescent probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, ThermoFisher Scientific, San Jose, California, USA) using a modification of the procedure described by (Malanga & Puntarulo, 1995). CM-H2DCFDA is a non-fluorescent molecule that freely permeates the plasma membrane and is hydrolyzed in the cytosol to form the DCFH carboxylate anion (Gomes *et al.*, 2005; Kalyanaraman *et al.*, 2012). Oxidation results in the formation of fluorescent DCF, which is maximally excited at 495 nm and emits at 520 nm (Kalyanaraman *et al.*, 2012) and can be measured by flow cytometry. For experiments, a fresh 1 mM stock solution was made immediately prior to use by dilution in 100% ethanol and a final concentration of 5 μM was used (Evans *et al.*, 2006). Cultures were incubated either in the presence of CM-H2DCFDA alone, with CM-H2DCFDA and 25 μg Cu L^{-1} or with CM-H2DCFDA and 250 μg Cu L^{-1}. These concentrations were selected after a preliminary experiment with strains RCC1216 and RCC1217 using the same Cu concentrations as for the enzyme detection experiments (0 and 100 μg Cu L^{-1}).

Triplicate analyses were made for all treatments, and samples were taken at 30, 60 and 90 min after the start of treatment for cytometer analysis recording green (530 nm) fluorescence of all red-fluorescent (chlorophyll-containing) cells.

Statistical analysis

Nominal IC_{50} values among strains were compared using a One-way Analysis of Variance (ANOVA) using the best-fit value obtained from the nonlinear regression (Mean=logIC_{50}; SEM=Standard Error and N=degrees of freedom+1, following the indications given by the GraphPad Software), followed by a Tukey's test for multiple comparisons. Differences for motility, PSII quantum yield, enzyme activity and ROS production were evaluated by One-way ANOVA followed by a Tukey's test for multiple comparisons. Percentage of normal coccolith morphotypes and incomplete/malformed coccoliths were compared using a Chi-square test. Differences in Cu-induced ROS production among strains was analyzed in two ways: the mean fold-increase in CM-H2DCFDA fluorescence (compared to stained but untreated control samples for each strain) was log2-transformed for performing a two-way ANOVA. The proportion of high CM-H2DCFDA fluorescent cells (>95th percentile of no Cu controls) was evaluated by running a two-way ANOVA followed by a Tukey's test for multiple comparisons (with a significance threshold $p<0.05$ after adjustment for multiple comparison). In both cases we focused on the intermediate time point of 60 min of Cu exposure. Similar trends were observed in both statistical tests of Cu-induced ROS accumulation, so for clarity only the first approach is presented. All analyses were conducted using Prism 6.0 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Effects of Cu on culture growth

Growth rates in control conditions after 96 h of culture during the toxicological bioassays, were highly variable among strains (Table 2). Compared to the mean growth rate in control conditions across all strains (0.598 ± 0.180 day^{-1}), the three Open Pacific strains showed lower growth rates (Table 2, average growth rate across the three strains was 0.367 ± 0.095 day $^{-1}$), while the diploid and haploid Mediterranean strains showed higher growth rates (0.760±0.032 and 0.883±0.028 d^{-1}, respectively). These differences resulted in the fact that for the same exposure time (e.g. 96 h) different numbers of generations were observed depending of the strain (Table 2). For IC_{50} results we present principally the results from the MinEx-IC_{50} calculations as it minimizes differences in Cu-response that may result from growth-rate differences, however, we note that the general patterns were similar for the two types of IC_{50} calculations. The strain CHC428 could not be included in the analysis as it did not reach the generation threshold.

Copper had an inhibitory effect on the growth for all strains with some important differences (see Table 3). The Mediterranean Sea strains F2 and B4 exhibited stronger sensitivity to Cu, with MinEx-IC_{50} values of 8.15 and 10.46 µg Cu L^{-1} , respectively. These values were significantly different (all cases $p<0.05$)

Table 2. Growth parameters of controls (0 µg Cu L^{-1}) for Emiliania huxleyi strains after 96 h of culture

	Mediterranean Sea		*Tasman Sea*			*Pacific Coast*				*Open Pacific*		
	F2	*B4*	*RCC1216*	*RCC1217*	*CHC350*	*CHC352*	*CHC366*	*CHC299*	*CHC307*	*CHC428*	*CHC440*	*CHC524*
Growth rate (d^{-1})	0.760 ±0.032	0.883 ±0.028	0.626 ±0.08	0.749 ±0.016	0.752 ±0.16	0.671 ±0.06	0.518 ±0.047	0.533 ±0.028	0.640 ±0.039	0.289 ±0.087	0.467 ±0.028	0.346 ±0.057
N° of generations	4.476 ±0.136	5.059 ±0.076	3.498 ±0.31	4.318 ±0.251	4.531 ±0.733	3.868 ±0.334	2.912 ±0.551	3.173 ±0.208	3.580 ±0.339	1.562 ±0.536	2.068 ±0.107	2.136 ±0.456

Average growth rate across the three strains: 0.367 ± 0.095 day^{-1}

Table 3. Response of strains to copper in growth inhibition bioassays

	Mediterranean Sea		*Tasman Sea*			*Pacific Coast*				*Open Pacific*		
	F2	*B4*	*RCC1216*	*RCC1217*	*CHC350*	*CHC352*	*CHC366*	*CHC299*	*CHC307*	*CHC428*	*CHC440*	*CHC524*
MinEx-IC50 (µg Cu L^{-1})	**8.15**	**10.46**	**12.44**	**41.74**	**22.45**	**14.81**	**16.69**	**24.24**	**50.87**	**-**	**12.35**	**15.45**
95% C.I. MinEx (µg Cu L^{-1})	6.58 - 10.10	6.71 - 16.31	11.24 - 13.77	35.40 - 49.21	15.55 - 32.41	11.87 - 18.48	9.48 - 29.40	9.253 - 63.51	24.13 - 107.3	-	10.52 - 14.49	12.79 - 18.66
Significant differences	b	b	b	ac	a	bc	bc	a	a		b	bc
Final time (h)	96	96	120	96	96	96	144	96	96	-	144	120
N° of generations	4.5	5.06	4.3	4.3	4.5	3.9	4.3	3.2	3.6	(<3)	3.5	3.0

Responses at MinEx-IC50 (see methods). C.I.: Confidence Intervals. Means that share one or more letters are not significantly different from each other according to the Tukey's test for multiple comparisons, $p > 0.05$.

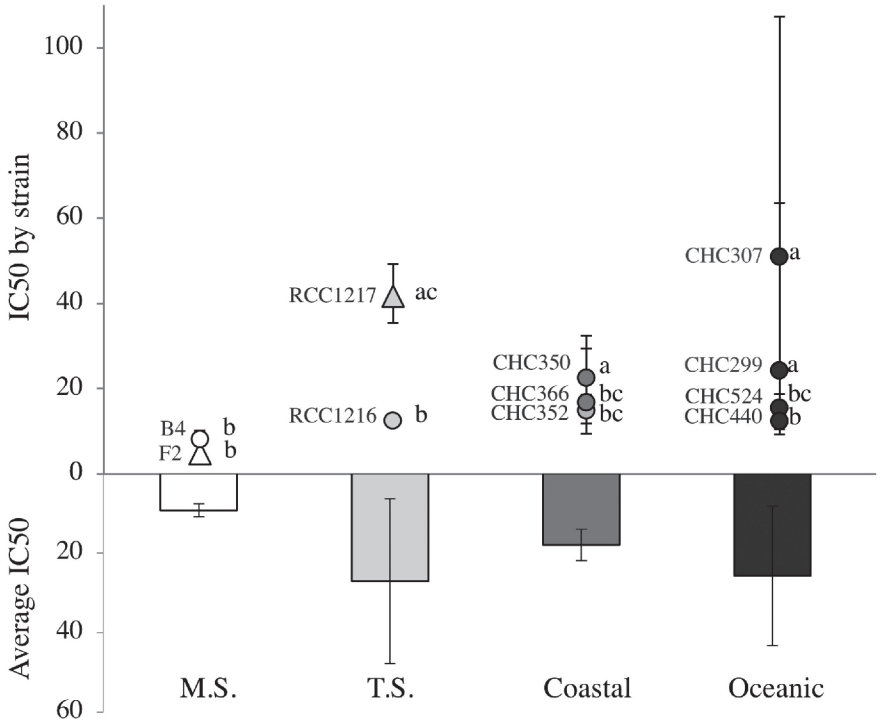

Fig. 1. MinEx-IC50 from every strain (**upper side)** and average MinEx-IC50 obtained in strains from Mediterranean Sea (M.S., white bar), Tasmanian Sea (T.S., light grey bar), coastal (dark grey bar) and oceanic (black bar) regions **(lower side)**. Data represents mean ± standard deviation (n = 3). Circles=diploid strains, triangle=haploid strains. A one-way ANOVA followed by Tukey posthoc test was performed to compare difference among strains ($F_{10, 209}$ = 8.947, p<0.0001). Means that share one or more letters are not significantly different from each other according to the Tukey's test (p>0.05).

from the more resistant strains, RCC1217, CHC350, CHC299 and CHC307, which showed MinEx-IC_{50} values over 20 µg Cu L^{-1} and up to 50.87 in the case of CHC307. Between those extremes, a range of sensitivities between MinEx-IC_{50} values of 12.35 and 16.69 µg Cu L^{-1} was observed for the rest of the strains, with no significant differences among them (p>0.05). No significant differences were observed when strains were grouped by oceanographic region, reflecting the wide variability observed in strains from same origin (Table 3, Fig. 1).

Tests using the live-dead stain FDA were performed in order to determine whether the variability among strains in sensitivity to growth inhibition by Cu might be due to differences in Cu-induced cellular mortality. For these tests we selected the diploid strain that displayed the highest sensitivity (F2) and a strain that displayed a lower sensitivity (RCC1216), as well as the haploid counterpart of RCC1216 (RCC1217) as it was among the most resistant strains we had tested. Results show that growth inhibition by Cu was not due to Cu-induced cell mortality, as there were no significant differences between controls and treatment with a high Cu concentration (100 µg L^{-1}) or among strains, with the percentage of cells judged to be viable always near 100% after 24 h exposure to high Cu (Fig. 2).

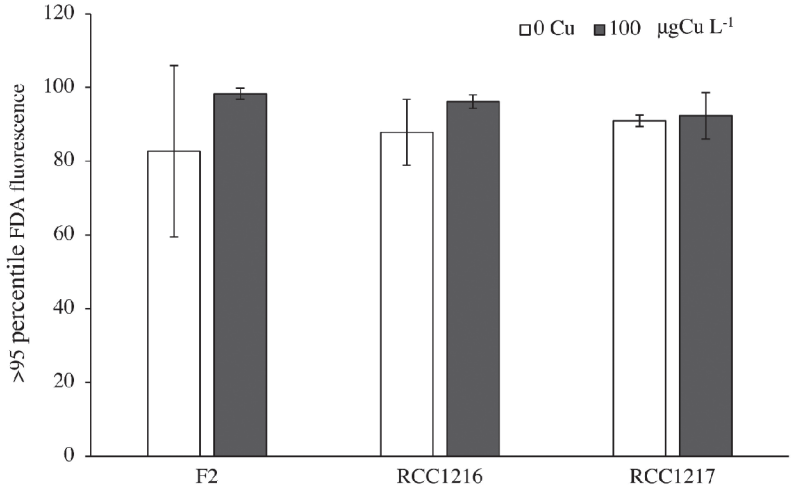

Fig. 2. Percentages of metabolically active cells (high FDA fluorescence) in three strains of *Emiliania huxleyi* after 24-h of exposure to 100 µg L^{-1} Cu. Bars represents mean ± standard deviation (n = 3). There were no significant effects of strain (p=0.94) or Cu-treatment (p=0.07) according to a two-way ANOVA.

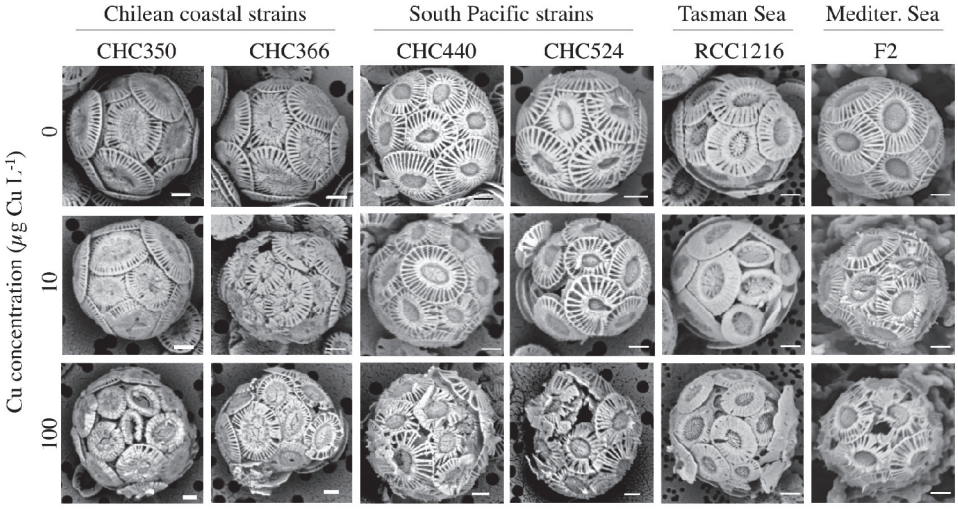

Fig. 3. External morphology of selected *Emiliania huxleyi* strains treated with 0, 10 and 100 µg Cu L^{-1} after 96 h of growth inhibition bioassays (Scale bars = 1 µm).

Cu-induced changes in coccolith morphology and motility

Copper had a severe effect on coccolith morphology in *Emiliania huxleyi* cells (Fig. 3 and 4). Cells of all strains exposed to 100 µg L^{-1} of Cu exhibited large numbers of incomplete and malformed coccoliths, while exposure to 10 µg L^{-1} Cu resulted in responses that were variable among strains (Fig. 3 and 4). Significant

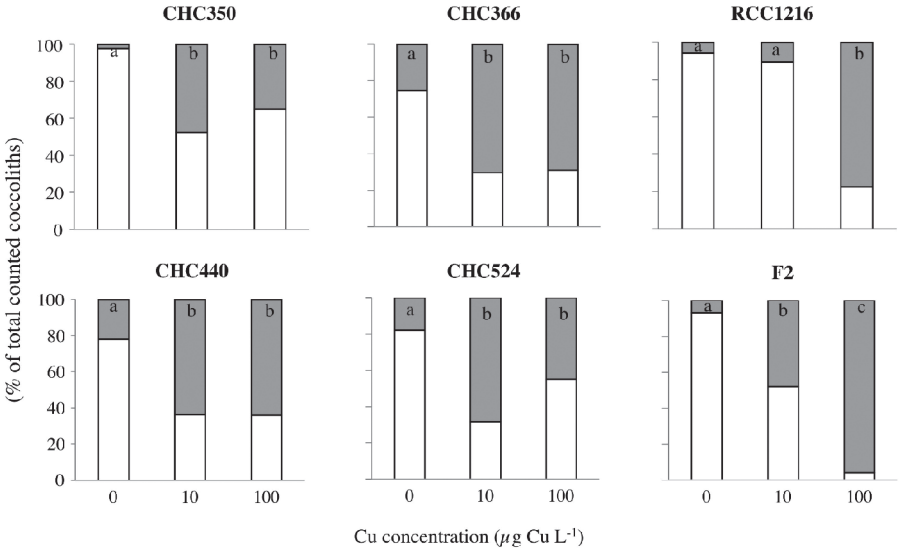

Fig. 4. Percentage of normal coccolith morphotypes (white) and incomplete/malformed coccoliths (grey) of selected *Emiliania huxleyi* strains treated with 0, 10 and 100 µg Cu L^{-1} after 96 h of growth inhibition bioassays. Means with different letters are significantly different (Chi-square test, $p< 0.01$).

differences were identified between control and the 100 µg L^{-1} Cu treatment for all strains. The F2 strain was the most affected, with almost 100% of coccoliths malformed or incomplete in the highest copper treatment (Fig. 3 and 4). Likewise, cells exposed to 10 µg L^{-1} of Cu exhibited significant differences from controls, with the exception of the RCC1216 strain, for which less than 15% of observed coccoliths were malformed or incomplete. Significant differences between both Cu treatments were observed only for RCC1216 and F2 strains. However, when all strains were included, no significant correlations were detected between the IC_{50} values for growth inhibition and the percentages of malformed coccoliths at either 10 µg L^{-1} or 100 µg L^{-1} ($p>0.05$, data not shown).

Copper exposure also strongly inhibited cell motility in the haploid stage (strain B4) (Fig. 5). Upon exposure to 100 µg L^{-1} of Cu, motility was almost completely absent. Although motility was somewhat less affected at 10 than 100 µg L^{-1} of Cu (≈80% of non-motile cells), this difference was not significant ($p>0.05$), suggesting that near maximal inhibition was achieved at the lower concentration.

Responses of variable fluorescence to Cu and peroxide stress

Effects of increasing Cu on PSII quantum efficiency (F_v/F_m) were not consistent among strains. For most of the strains tested (exemplified by RCC1217 shown in Fig. 6; upper panel), no effects of Cu were detected even at the highest concentration tested (100 µg L^{-1}). However, high concentrations of Cu (45 and 100 µg L^{-1}) did cause a significant drop in two strains (ca. 50%), which included CHC350 of coastal origin (Fig. 6; central panel), and the open ocean strain CHC299.

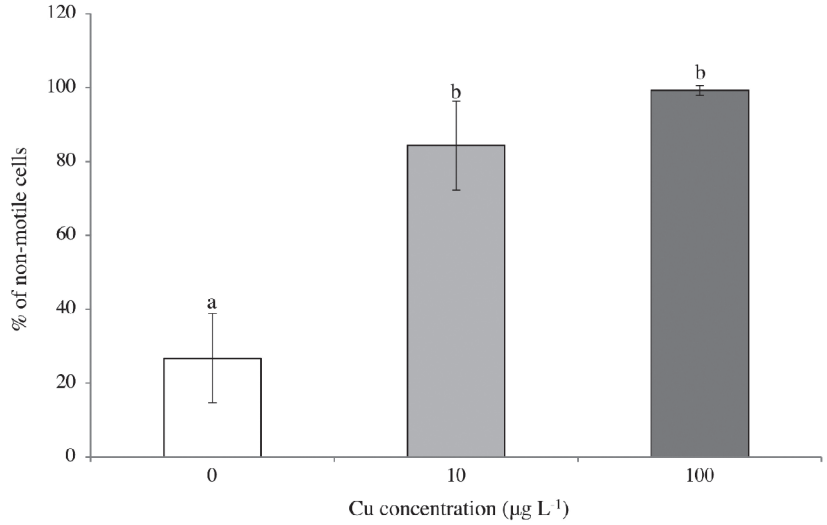

Fig. 5. Percentage of non-motile cells of B4 *Emiliania huxleyi* strain after 96 h of growth inhibition bioassays with 0, 10 and 100 µg Cu L^{-1}. Data represents mean ± standard deviation (n = 3). A one-way ANOVA followed by Tukey posthoc test was performed to compare difference between strains ($F_{2, 6}$ = 45.07, p<0.0005). Means that share one or more letters are not significantly different from each other according to the Tukey's test (p>0.05).

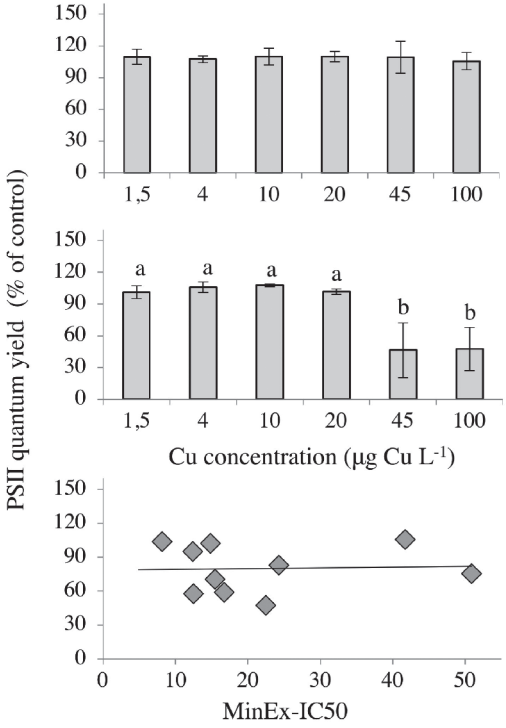

Fig. 6. Maximal PSII quantum yield (Fv/Fm, as % of controls) at the end of Cu bioassays of growth of RCC1217 **(upper panel)** and CHC350 **(central panel)**. (**Lower panel):** Correlation between MinEx-IC50 results from the copper bioassays and maximal quantum yield (Fv/Fm, calculated as percentage of control values) obtained in presence of 100 µg Cu L^{-1} at final time of the copper bioassays (r^2=0.00195; p=0.903). For a and b data represents mean ± standard deviation (n = 3). A one-way ANOVA followed by Tukey posthoc test was performed to compare difference between strains ($F_{5, 11}$ = 15.51, p<0.0001). Means that share one or more letters are not significantly different from each other according to the Tukey's test (p>0.05).

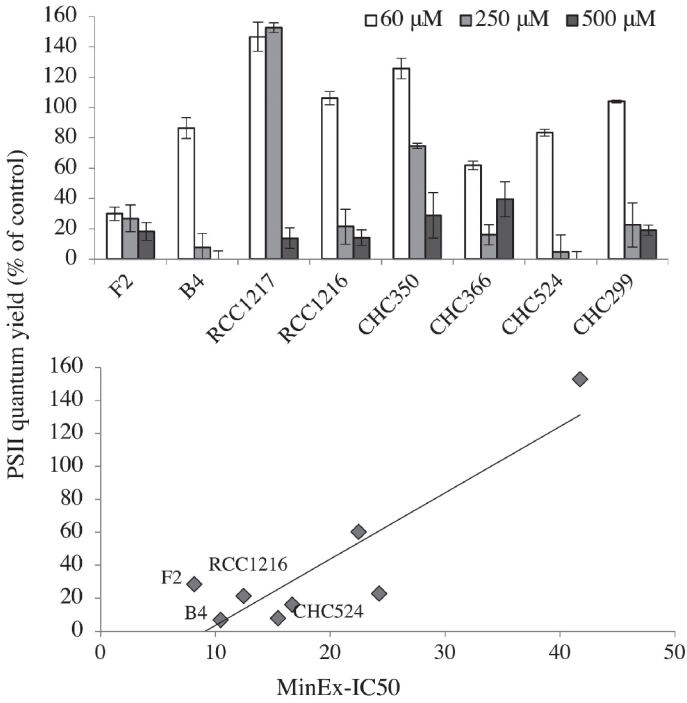

Fig. 7. Maximal PSII quantum yield (Fv/Fm, as % of controls) of selected strains after 30 min of exposure to 60, 250 and 500 µM of H_2O_2 **(upper panel)**. Data represents mean ± standard deviation (n = 3). Correlation between MinEx-IC50 from the copper bioassays of *Emiliania huxleyi* strains and the Photosystem II maximal quantum yield (Fv/Fm, calculated as percentage of control values) obtained after 30 min of exposure to 250 mM of H_2O_2 **(lower panel)** (r^2=0.78499; p=0.003).

When all strains were considered, there was no correlation between growth inhibition by Cu and the effect on F_v/F_m (Fig. 6; lower panel).

To rule out that the possibility that the lack of a consistent Cu response of F_v/F_m seen in long-term growth experiments was the result of acclimation, we selected the most sensitive diploid strain (F2) and the moderately resistant diploid strain (RCC1216) for testing the effects of short-term exposure. Although treatment with 100 µg L^{-1} Cu induced a modest initial drop in F_v/F_m compared to the control within the first 2 h (to 87±0.74% in F2 and 95%±1.16% in RCC1216), by 24 h there was no difference between Cu-treated and control samples in either strain (p>0.0; data not shown).

Variable fluorescence was diminished by 30 min exposure to H_2O_2. When cells were exposed to 60 µM H_2O_2, some strains were resistant, while exposure to 500 µM H_2O_2 resulted in a strong decrease in F_v/F_m. At a dose of 250 µM H_2O_2, an effect on the PSII quantum efficiency was observed for all strains except RCC1217 (Fig. 7; upper panel). A positive correlation was observed between the MinEx-IC_{50} values from growth inhibition experiments and F_v/F_m obtained after 30 min of exposure to 250 mM of H_2O_2 (Fig. 7; lower panel).

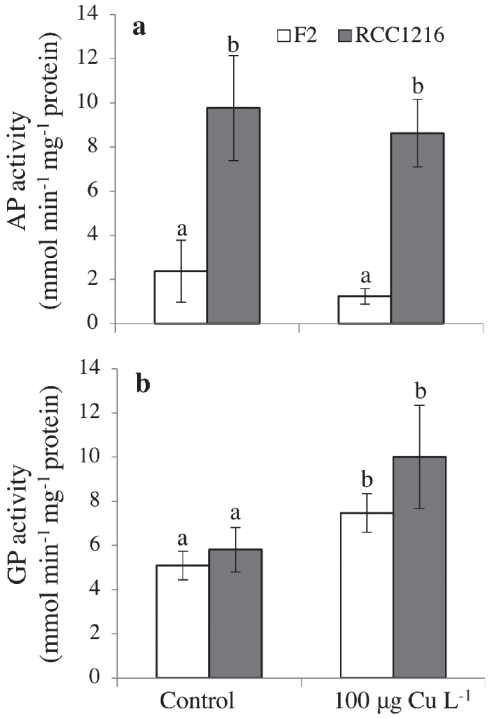

Fig. 8. Activities of the antioxidant enzymes Ascorbate peroxidase (AP, **upper panel**) and Glutathione Peroxidase (GP, **lower panel**) for F2 (white bars) and RCC1216 (grey bars) *Emiliania huxleyi* strains exposed to control medium and 100 µg Cu L^{-1}. Data represents mean ± standard desviation (n = 3). For each of the figures, a one-way ANOVA followed by Tukey posthoc test was performed to compare difference between strains and treatments (AP: $F_{3, 8}$ = 22.16, p<0.0005; GP: $F_{3, 8}$ = 7.421, p<0.05). Means that share one or more letters are not significantly different from each other according to the Tukey's test (p>0.05).

Basal and Cu-induced antioxidant enzyme activities

Antioxidant enzyme activities were compared between the most sensitive diploid strain F2 and the moderately resistant diploid strain RCC1216. Basal AP activity was approximately 4-fold higher in the moderately resistant strain RCC1216 compared to the sensitive strain F2. However, there was no further induction of AP-activity after 24 h of Cu exposure (Fig. 8; upper panel). Basal GP activity was similar between the two strains, and was increased by ca. 50% after 24 h of Cu exposure (Fig. 8; lower panel).

We were not able to perform this assay on haploid cells. Preliminary experiments showed large losses of the non-calcified haploid cells during initial the centrifugation step. We attempted filtration, but the comparatively higher vacuum pressures and times needed to concentrate haploid cells sufficiently raised the concern that some cell lysis of these unprotected cells would lead to results not being comparable to those of calcified diploid cells.

Changes in the ROS levels as response to acute exposure to excess Cu

We tested whether the differences in sensitivity to growth inhibition by Cu reflect differences in the sensitivity to acute Cu-induced ROS accumulation. For this experiment, we selected the diploid strains F2 and RCC1216 and the corresponding haploid strains B4 and RCC1217, as strain F2 exhibited the highest sensitivity to

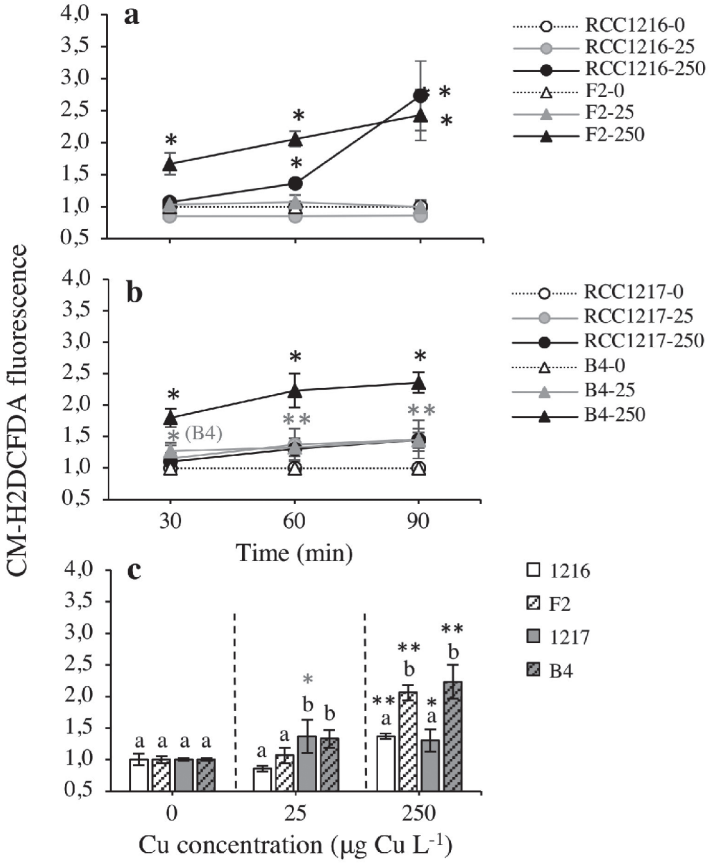

Fig. 9. Time courses of 0, 25 and 250 Cu µg L^{-1}-induced ROS in the diploid (**upper panel**) and haploid (**central panel**) strains. For each strain, a two-way ANOVA followed by Tukey posthoc test was used to determine when 250 µg L^{-1} (black *) or 25 µg L^{-1} (grey *) Cu treated samples were different from controls. Results of a full multiple pairwise comparison is provided in Table 4. A two-way ANOVA followed by Tukey posthoc test was performed with all data at the 60 min time point to test for differences among strains **(lower panel).** The effects of strain, dose, and the interaction of these factors were significant ($F_{3,24}$=16.6, p<0.001; $F_{2,24}$=98.6, p<0.001; $F_{6,24}$=11.9, p<0.001). Shared letters indicate when the responses of two or more strains were not significantly different within a given Cu treatment in pairwise treatments, while * indicate differences among treatments within a strain (grey *, 25 µg L^{-1} Cu different from control; black *, 250 µg L^{-1} Cu different from control; **, 250 µg L^{-1} Cu different from 25 µg L^{-1} Cu and control).

growth inhibition by Cu while strain RCC1217 exhibited among the highest resistance to Cu. Concentrations of 25 µg Cu L^{-1} (as intermediate among the IC_{50} values for growth inhibition) and 250 µg Cu L^{-1} (to be one order of magnitude higher) were selected. Cu-induced ROS-accumulation varied depending on concentration, time, and strain (Fig. 9, Table 4). In diploid strains (RCC1216 and F2), the high concentration of Cu (250 µg L^{-1}) induced ROS accumulation after 90 min, but not at lower concentrations (25 µg L^{-1}). Strain F2 accumulated ROS faster than RCC1216 within the first 60 min (Fig. 9; upper and lower panels and Table 4).

However, at 90 min the intracellular ROS levels were similar between strains (Fig. 9; upper panel and Table 4). In contrast to the diploid life stages, acute exposure to both 25 and 250 µg L^{-1} Cu induced ROS accumulation in the haploid stage (Fig. 9; central and lower panels). Notably, mean ROS accumulation in response to the highest concentration of Cu was much lower in the Cu-resistant strain RCC1217 than in the Cu-sensitive strain B4.

DISCUSSION

Emiliania huxleyi has been previously shown to be resistant to Cu toxicity compared to other coccolithophores (Brand *et al.,* 1986), and coccolithophores in general showed an intermediate level of resistance, with cyanobacteria and dinoflagellates highly sensitive while some coastal diatoms and green algae are highly resistant (Brand *et al.*, 1986; Levy *et al.*, 2007). Here we found a > 5-fold variation within *E. huxleyi* in sensitivity to inhibition of growth by Cu, with sensitivity varying by both among genetic backgrounds and between life stages of the same genetic backgrounds (Table 3, Fig. 1). Our objective in this study was to understand intra-specific variability, rather than to establish absolute toxicological sensitivities to Cu for comparison to environmental values. However, we note that the range of sensitivities observed were within the range of values previously reported for this species (Levy *et al.,* 2007; Echeveste *et al.,* 2018), despite methodological differences among studies (e.g., use of natural versus artificial seawater, or glassware versus plasticware experimental vessels). The three strains isolated from coastal South Pacific waters were not more resistant than five strains isolated from oceanic waters, in agreement with the observations made by Echeveste *et al.*, where no significant differences in sensitivity to Cu were observed when coastal and offshore strains were compared. Unlike the South Pacific open ocean, where Aeolian inputs of metals are minimal, the Mediterranean receives Aeolian input of Cu that has been shown to inhibit phytoplankton growth (Jordi *et al.*, 2012), yet both life cycle stages of the one Mediterranean strain tested exhibited the least resistance to Cu. Thus, variability in sensitivity to Cu did not correlate in any clear way with geographic origin. These results are in contrast to *Synechococcus,* highly Cu-sensitive picophytoplankton, where the sensitivity to Cu has been reported to correlate with Cu inputs (Stuart *et al.* 2013).

In both cases where alternant life stages from the same genetic background were tested, the haploid (non-calcifying) stage was more resistant than the diploid (calcifying) stage (Table 3, Fig. 1). A previous study also observed that parent calcifying strains were more sensitive than daughter strains that were non-calcifying in both *Emiliania huxleyi* and its close relative *Gephyrocapsa oceanica* (Levy *et al.*, 2007), although they did not mention whether the non-calcifying strains were haploids or the diploid non-calcifying forms that also frequently appear (Klaveness & Paasche, 1971; Klaveness, 1972), including apparent non-calcifying diploid mutants (Mackinder *et al.*, 2011). As noted by Levy *et al.* (2007), a higher sensitivity of calcified strains is surprising, because the inorganic coccoliths provide a large inert surface area would be expected to adsorb Cu, potentially reducing bio-available Cu at the cell surface. However, although haploid *E. huxleyi* are not calcified, they are covered in organic scales. In addition, coccolithogenesis must be supported by very high ion fluxes (Mackinder *et al.*, 2010), and the calcified stage has higher

Table 4. Results of multiple pairwise comparison of strains, time, and Cu dose on ROS accumulation (mean CM-H2DCFDA fluorescence). Shown are significance (p-value threshold <0.05) after correction for multiple comparison (Tukey test). ns = not significant.

		Strain	*1216*		*F2*			*1217*			*B4*		
Time (min)	*Strain*	*[Cu]*	*25*	*250*	*0*	*25*	*250*	*0*	*25*	*250*	*0*	*25*	*250*
30	1216	0	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		25		ns	ns	ns	<0.0001	ns	ns	ns	ns	ns	ns
		250			ns	ns	<0.0001	ns	ns	ns	ns	ns	<0.0001
	F2	0				ns	<0.0001	ns	ns	ns	ns	ns	<0.0001
		25					<0.0001	ns	ns	ns	ns	ns	<0.0001
		250						<0.0001	0.0011	0.0002	<0.0001	ns	ns
	1217	0							ns	ns	ns	ns	<0.0001
		25								ns	ns	ns	<0.0001
		250									ns	ns	<0.0001
	B4	0										ns	<0.0001
		25											0.005
60	1216	0	ns	0.0172	ns	ns	<0.0001	ns	0.0265	ns	ns	ns	<0.0001
		25		<0.0001	ns	ns	<0.0001	ns	<0.0001	0.0003	ns	0.0001	<0.0001
		250			0.0182	ns	0.0004	0.0188	ns	ns	0.0188	ns	<0.0001
	F2	0				ns	<0.0001	ns	0.0281	ns	ns	ns	<0.0001
		25					<0.0001	ns	ns	ns	ns	ns	<0.0001
		250						<0.0001	0.0003	<0.0001	<0.0001	<0.0001	ns
	1217	0							0.0289	ns	ns	ns	<0.0001
		25								ns	0.0288	ns	<0.0001
		250									ns	ns	<0.0001
	B4	0										0.0582	<0.0001
		25											<0.0001

		Strain		*1216*			*F2*			*1217*			*B4*
Time (min)	*Strain*	*[Cu]*	*25*	*250*	*0*	*25*	*250*	*0*	*25*	*250*	*0*	*25*	*250*
90	1216	0	ns	<0.0001	ns	ns	<0.0001	ns	0.0028	0.0027	ns	0.0026	<0.0001
		25		<0.0001	ns	ns	<0.0001	ns	<0.0001	<0.0001	ns	<0.0001	<0.0001
		250			<0.0001	<0.0001	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	ns
	F2	0				ns	<0.0001	ns	0.0026	0.0025	ns	0.0023	<0.0001
		25					<0.0001	ns	0.0023	0.0022	ns	0.0021	<0.0001
		250						<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	ns
	1217	0							0.0029	0.0028	ns	0.0026	<0.0001
		25								ns	0.003	ns	<0.0001
		250									0.0029	ns	<0.0001
	B4	0										0.0027	<0.0001
		25											<0.0001

expressions of transporters related to Ca^{2+}, H^+, HCO_3^-, Na^+, K^+ and Cl^- than the non-calcifying stages (von Dassow *et al.*, 2009; MacKinder *et al.*, 2011; Taylor *et al.*, 2011), which might result in higher fluxes of all ions (including Cu). However, Levy *et al.* (2007) also tested an isolate of *Isochrysis* sp. a close relative of *E. huxleyi* and *G. oceanica* that appears to be stuck in the non-calcifying haploid phase (and exhibits the same scales and flagellar structure as *E. huxleyi* haploid cells, Carrier *et al.*, 2014), and found it to be much more sensitive than the calcified stages of *E. huxleyi* and *G. oceanica*. Thus the observed higher resistance to Cu of the haploid life stage in *E. huxleyi* might reflect a species-specific adaptation of this life stage to resist certain types of stresses, emphasizing that the ecological role of the life cycle in this species remains unknown.

We note that the Cu concentrations used in these experiments were determined to be sub-lethal by FDA staining (Fig. 2). Likewise, effects of Cu on photosynthetic efficiency (F_v/F_m) were not detected in most strains. The use of modern pulsed amplitude modulated or fast repetition fluorometers (which were not available to us during this study) might have been able to detect more subtle effects, perhaps on other parameters of photosynthetic electron transport. However, in initial tests our method successfully showed the dose-dependent drops and recoveries of F_v/F_m in response to H_2O_2-treatment, showing that we could successfully detect changes in functioning of the photosynthetic electron transport system (Fig. 7; upper panel and data not shown). Also, our results are in agreement with several other studies in different phytoplankton and macroalgae that also have observed that F_v/F_m and other parameters of variable fluorescence tend to be less sensitive to Cu-toxicity than growth (Juneau *et al.*, 2002; Miller-Morey & Van Dolah, 2004; Perales-Vela *et al.*, 2007; Lombardi & Maldonado, 2011). More importantly, the two strains in which a decrease in F_v/F_m was observed in response to high Cu were strains which had exhibited intermediate sensitivity to growth inhibition. This suggests that sensitivity to growth inhibition by Cu did not reflect a sensitivity of photosynthetic electron transport to Cu-toxicity.

Growth inhibition is the most commonly tested parameter for toxicity studies, but other biological outputs can also be affected. Here we found that Cu toxicity strongly negatively affected coccolithogenesis of diploids (Fig. 3 and 4). In diploids, Cu treatment resulted in the production of incomplete and malformed coccoliths. Curiously there was no overall correlation between the Cu-sensitivity of growth and the Cu-sensitivity of coccolithogenesis.

Growth inhibiting concentration of Cu also strongly inhibited motility in haploids (Fig. 5). We could only test the effect on motility of one of the haploids (the most Cu-sensitive strain) due to that the percentage of motile cells in the most Cu-tolerant strain was low under control conditions during the course of the study. However, it is noticeable that motility was >80% inhibited at 10 $\mu g\ L^{-1}$ nominal Cu, the concentration that caused only a 50% inhibition of growth. Thus, motility might be more sensitive than growth.

High Cu is well known to result in oxidative stress, and resistance to Cu in other algae involves the production of anti-oxidant enzymes (Maksymiec, W. 1997; Correa *et al.,* 1996; Contreras *et al.,* 2009). Likewise, we observed that acute exposure to high Cu caused a very rapid accumulation of ROS inside the cells (Fig. 9). Notably, for both diploid and haploid stages, the Cu-resistant genetic background appeared to accumulate intracellular ROS more slowly or to lower levels than the Cu-sensitive genetic background in response to Cu. This suggested that the difference in tolerance to Cu among diploids might be due to differences in the capacity to tolerate oxidative stress.

GP activity was responsive to Cu, as has been seen in other algae (Jervis *et al.*, 1997; Pinto *et al.*, 2003; Contreras *et al.*, 2005; Contreras *et al.*, 2009) although no significant differences were observed between the two strains with different Cu-tolerances (Fig. 8). AP activity did not increase in response to Cu. However, the constitutive activity of this enzyme was substantially different between the Cu-sensitive strain F2 and the moderately Cu-tolerant strain RCC1216. This indicates that part of the differences among strains may relate to intra-specific differences in anti-oxidant activities. It is worth noting that Cu induces production and exudation of Gln-Cys (a precursor to GSH) and Arg-Cys thiols, which form extracellular complexes with Cu in *Emiliania huxleyi* (Dupont *et al.*, 2004). GSH is the substrate for phytochelatin synthase (reviewed in Kawakami *et al.*, 2006; Masmoudi *et al.*, 2013), so Cu-induced GP activity might also work against phytochelatin production. This might help explain the previously reported observation that Cu-induced accumulation of intracellular phytochelatin in *E. huxleyi* dropped at high Cu exposures (Ahner *et al.*, 2002).

Although we were not able to test enzyme activities on the haploid strains using the same protocol as used for diploid strains, the observation that F_v/F_m was not diminished by peroxide treatment in RCC1217 and that acute Cu induced only low ROS accumulation in this strain suggests that it might have higher constitutive antioxidant activities than the other strains tested. It should be noted that as Cu concentration was not measured along the experiments and they can not been calculated from the available data, it is likely that nominal Cu concentrations do not represent the bio-available fraction of this metal. Under this scenario, we can interpret that GP activity was responsive to Cu and that constitutive AP activity differed between strains but we cannot rule out that AP activity might be responsive to other concentrations of Cu (or at other times of exposure), nor can we rule out that the differences in Cu-induced GP activity between strains might occur if we had been able to test lower cell densities and to better control the actual exposure concentration.

Although here we have focused on oxidative stress responses, it is important to recognize that the relatively high tolerance of *Emiliania huxleyi* to Cu likely relates in part to its production of Cu-chelating ligands such as thiols (Dupont *et al.,* 2004; Leal *et al.,* 1999; Vasconcelos *et al.,* 2001; Echeveste *et al.*, 2017). The production of these ligands is both spontaneous and further induced by Cu. The extrusion of these Cu-binding ligands may also be the mechanism for a very high Cu export rate from *E. huxleyi* cells (Walsh & Ahner, 2014). In a recently published work authors explore how production of organic ligands could explain differences in sensitivity of *E. huxleyi* to Cu-inhibition of growth. The results of the work show that the variability in Cu-sensitivity was at least partially explained by the production of organic Cu-ligands, being the most productive strains the most tolerant to Cu at constitutive levels (Echeveste *et al.*, 2018). However, the fact that in our experiment there were no correlations of Cu-sensitivities among different biological processes (growth, coccolithogenesis, and photosynthetic electron transport efficiency) suggested that differences in Cu-ligand production and Cu-export might not be sufficient to explain the observed variability of sensitivity to Cu-toxicity observed among strains: Differences in a mechanism that diminishes Cu-availability outside the cell or the total Cu concentration inside the cell would be expected to result in a strong co-variation between growth inhibition and negative effects on other cellular and sub-cellular processes. This consideration supports our conclusion that intracellular mechanisms also contribute to Cu-resistance. Therefore, the intra-specific variability we observed is likely to be found in sensitivity to broad classes

of environmental stressors beyond Cu toxicity, including reactivity to other metals or other factors that induce oxidative stress.

In summary, our results show high intra-specific variability in tolerances to Cu among genetic backgrounds and life cycle stages in the cosmopolitan species *Emiliania huxleyi*. However, the sensitivity to Cu also varied depending on what biological process was measured. In general, tolerance to Cu did not vary by geographic origin but the haploid phase tends to be more resistant to this stress than the diploid phase. The intra-specific variability in sensitivity to Cu might be partly explained by differences in more general abilities to respond to oxidative stress.

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