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**Species-species interactions modulate copper toxicity under different
visible light conditions**

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Abstract

Combination of biotic and abiotic factors influence the effects of naturally occurring or anthropogenic chemicals to photosynthetic microorganisms in the aquatic environment. Nonetheless, the combined effects of physical stressors and specie-specie interaction on chemicals toxicity are still poorly understood. The present study examines the responses of the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* sp. alone and in mixtures to copper exposure under increasing visible light intensities. Cell growth, chlorophyll bleaching, oxidative stress and membrane permeability were determined by flow cytometry in both mono-and multispecies algal tests. The results revealed that specie-specie interactions influenced copper toxicity under different light regimes at 4h and 48h- exposure. For a given light condition, monocultures of *Synechocystis* sp. were more sensitive to copper than *C. reinhardtii*. In long term incubation *C. reinhardtii* sensitivity to copper diminished in presence of *Synechocystis* sp. under low-intensity light, however was enhanced under high-intensity light. The present results revealed the complex interplay between visible light intensity variations, specie-specie interaction and copper effects to phytoplankton in long term-exposure.

Keywords: Specie-specie interaction; Copper; Light irradiation; Multiple stressors; *Chlamydomonas*; *Synechocystis*.

33 **Highlights**

- 34 • Dissimilar influence of specie-specie interaction on Cu toxicity under different light
- 35 conditions
- 36 • *Synechocystis* sp. reduces *C. reinhardtii* sensitivity to Cu at low-light conditions
- 37 • *Synechocystis* sp. increases *C. reinhardtii* sensitivity to Cu at high-light conditions
- 38 • *C. reinhardtii* affectes *Synechocystis* sp. response to Cu at short-term incubation
- 39 • *Synechocystis* sp. is more sensitive to Cu than *C. reinhardtii* in single specie tests

40

1. Introduction

In the environment phytoplankton species are known to interact through nutrient and light competition (Passarge et al., 2006) as well as through allelochemical or secondary metabolite production (Leflaive and Ten-Hage, 2007). However, the naturally occurring or anthropogenic chemicals might alter specie-specie interactions and thus have indirect effects at the community level (Clements and Rohr, 2009). Conversely, the specie-specie interactions could be also expected to affect the sensitivity and tolerance of the microorganisms to chemicals (De Laender et al., 2009). Nevertheless, very little is still known about the role of such specie-specie interactions as modulators of chemical toxicity to photosynthetic microorganisms in the aquatic environment, although their important environmental relevance. Indeed, very few studies exploring the effects of the specie-specie interactions on contaminant toxicity in multispecies tests revealed contrasting findings. An increase of copper toxicity to euglenid *Trachelomonas* sp. in co-cultures with cyanobacterium *Mycrocystis aeruginosa* and green alga *Pseudokirchneriella subcapitata* (Franklin et al., 2004) was found. By contrast a decrease in copper toxicity to *M. aeruginosa* was observed in presence of other green algae *Chlorella pirenoidosa* and *Scenedesmus obliquus* (Yu et al., 2007). The effect of the flame retardant tetrabromobisphenol A on diatom *Nitzschia palea* was significantly higher in co-culture with *P. subcapitata* in comparison with the single-specie test (Debenest et al., 2011). Finally, pentachlorophenol led to a significant growth promotion of the cyanobacterium in a mixture of *M. aeruginosa* and *C. vulgaris* (de Morais et al., 2014). Beneficial effects of the interspecies interactions of algae *Isochrysis galbana* and *Platymonas subcordiformis* in response to Ciprofloxacin hydrochloride were very recently demonstrated (Wang et al., 2017).

What is more, the influence of multiple stressors (e.g. the interplay of visible light and chemicals (Cheloni et al., 2014)) on photosynthetic aquatic organisms in multi-species tests is not explored. Light is one of the highly variable parameters in the aquatic environment with intensities ranging from limiting to saturating for the photosynthetic organisms (Davies - Colley et al., 1984; Penta et al., 2008). Visible light might, at the same time, influence the physiological functioning of the organisms and might induce a stress when its intensity exceeds the photosynthetic capacity of the organism (Li et al., 2009). Furthermore, short wavelength radiation (e.g. UVR) induced detrimental effects to aquatic photosynthetic organisms (Hader et al., 2015; Roy, 1993), as well influenced the toxicity of naturally occurring or anthropogenic chemicals to photosynthetic organisms (Cheloni et al., 2014; Cheloni et al., 2016; Fischer et al., 2010; Korkaric et al., 2015a; Korkaric et al., 2015b; Nielsen and Nielsen, 2010; Regier et al., 2015; Xu and Juneau, 2016) and communities (Corcoll et al., 2012; Navarro et al., 2008). Nonetheless, the majority of these works deals with UVR and showed significant interactions, resulting in a reduction or an enhancement of the chemical toxicity depending on the light intensity and spectral composition (e.g. ratio visible/UVR) (Cheloni et al., 2014; Cheloni et al., 2016), as well as on the mode of toxic action (Korkaric et al., 2015a) and on the ability of the single organism (Korkaric et al., 2015b) or communities (Navarro et al., 2008) to acclimate to UVR. Few studies demonstrated that visible light might decrease or increase the sensitivity to the chemicals depending on the light intensity (Fischer et al., 2010).

In such a context, the present work examines the responses of the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* sp., alone and in mixed cultures, to copper exposure under visible light of increasing intensity. The strains were chosen

to represent two distinct phylogenetic groups that have different sensitivities to copper (Huertas et al., 2014; Jamers et al., 2013). In addition they are characterized by different high-light sensing-responding pathways (Mulo et al., 2012). To distinguish between *C. reinhardtii* and *Synechocystis* sp. populations in the multispecies tests flow cytometry (FCM) was used. FCM is a very promising technique for toxicity studies with microalgae because of its ability to provide simultaneously data about the different cellular responses of multiple populations present in the same culture (Franklin et al., 2004; Yu et al., 2007).

2 Material and methods

2.1 Test organisms and culture conditions

Chlamydomonas reinhardtii CPCC11 (Canadian Phycological Culture Centre, Department of Biology, University of Waterloo, Canada) and *Synechocystis* sp. PCC6803 (Pasteur Culture collection of Cyanobacteria, Institute Pasteur, France) were axenically grown in a modified BG11 medium (Rippka et al., 1979). This medium contained NH_4NO_3 as N-source instead of NaNO_3 and FeCl_3 instead of ammonium iron citrate. pH was maintained at 7.5 using 1 mM 3-(N-morpholino)propanesulfonic acid (MOPS, Sigma-Aldrich, Buchs, Switzerland). Algae were cultivated in a specialized incubator (Infors, Bottmingen, Switzerland) at 20°C, under 45 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 16:8 light cycle under rotary shaking at 110 rpm. Cell growth and cellular traits (cell size, chlorophyll and phycocyanin fluorescence) were routinely monitored all along the cell cultivation.

2.2 Copper exposure of mono- and multispecies cultures under different light regimes

Mono- and multialgal cultures acclimated to low-intensity light were exposed to CuSO₄ concentrations increasing from 0.2 to 12 µM and incubated under visible light of increasing intensity for 48 h (PAR45, PAR215 and PAR395). Exposures were performed in Erlenmeyer flasks, cultures had a total volume of 50 mL and were incubated in a specialized shaker under constant temperature (20°C) and agitation (100 rpm). Copper was added as CuSO₄ from 1 mM and 100 µM stock standard solutions (AAS grade, Sigma Aldrich). The inoculum of *C. reinhardtii* and *Synechocystis* sp. in mono- and multialgal culture experiments contained cells with equivalent surface area of $1.1 \times 10^7 \mu\text{m}^2/\text{mL}$ corresponding to 1×10^5 and 9.06×10^5 cell/mL for *C. reinhardtii* and *Synechocystis* sp., respectively (Table S1). To determine the effects of visible light intensities on Cu toxicity in mono- and multialgal cultures were incubated under three different conditions: low-intensity visible light, commonly used in microalgal toxicity testing (PAR 45 µmol photons m⁻² s⁻¹), a medium-intensity light (PAR 215 µmol photons m⁻² s⁻¹) and a high-intensity light (PAR 395 µmol photons m⁻² s⁻¹). The exposure duration of 48 hours was chosen to avoid confounding influence of nutrient starvation on the algal growth under longer exposure (Figures S1 and S2).

2.3 Biological endpoint measurement

The following endpoints were determined after 4h and 48 incubation to assess the effects of Cu and light: growth inhibition (GI), chlorophyll fluorescence bleaching (CB), intracellular oxidative stress (OS) and membrane damage (MD). These endpoints were chosen based on the known mode of action of copper and high-intensity light, as well as our previous results (Cheloni et al., 2014).

Under different exposure conditions, the cell number, chlorophyll fluorescence, intracellular oxidative stress and membrane damage were determined by FCM using a BD Accuri C6 (Accuri cytometers Inc., Michigan). The flow cytometer is equipped with a blue light (488 nm) excitation laser and three detectors for determination of green (530 ± 15 nm), yellow (585 ± 20 nm) and red (670 ± 25 nm) fluorescence. To this end 1 mL algal suspensions were collected after 48h-exposure and passed through FCM using the medium flow rate. The threshold was set to 20000 events. Data acquisition and analysis was performed with BD Accuri C6 Software 264.15. The gating strategy to discriminate between *C. reinhardtii* and *Synechocystis* sp. populations is provided in Figure S3.

Growth inhibition and bleaching of chlorophyll fluorescence were determined by direct cell counting and following the variations in the chlorophyll autofluorescence in mono- and multi-algal cultures. The percentage of cells with intracellular oxidative stress and with damaged membranes were determined using the fluorescent probes CellROX[®]Green (Life Technologies Europe B.V., Zug, Switzerland) and propidium iodide (PI, Sigma–Aldrich, Buchs, Switzerland), following the previously developed methodology (Cheloni et al., 2014).

The initial free copper ion concentrations ($[Cu^{2+}]$) in the exposure medium were calculated by visual MINTEQ version 3.0 (Gustafsson). Dose-response curves were obtained plotting the selected biological endpoint vs $[Cu^{2+}]$, thus the reported values for effect concentrations inducing response of 5 or 50% of the population (EC5 or EC50) refer to $[Cu^{2+}]$. To enable comparison with results from the literature, EC5 and EC50 values were also expressed in total copper concentration (Tables S2-S5).

2.4 Statistical analysis and data treatment

Statistically significant differences between monoalgal and mixed cultures were tested using Mann-Whitney Rank Sum Test ($p < 0.05$) in Sigma Plot 11.0. Concentration-response curves for different biological endpoints were fitted using a four parameter log-logistic model to determine Cu concentrations inducing effect in 5% and 50% of cell population. EC5s and EC50s with 95% confidence intervals (CI) obtained for each biological endpoint and their respective Hill slopes are given in Tables S2-S5. Significant differences among the treatments were assumed when the EC5 and EC50 values and their 95% CI were not overlapping (Korkaric et al., 2015b).

3. Results and discussion

3.1 Copper toxicity in monoalgal cultures under different visible light irradiation

Algal response to copper were first tested in monospecies cultures supplemented with increasing Cu concentrations under several visible light treatment conditions PAR45, PAR 215 and PAR395. After 4 hours incubation no effects were observed in *C. reinhardtii* cultures (Table S2) whereas short-term effects on oxidative stress, membrane permeability and chlorophyll bleaching could be observed in *Synechocystis* sp. mono-cultures (Figure 1). Furthermore, Cu effects on oxidative stress were enhanced in presence of high-light irradiations (Figure 1), but no significant differences were observed for the other end points. Among the tested long term biological responses, growth inhibition was the most sensitive followed by oxidative stress, membrane damage and chlorophyll bleaching (Figures 2 and 3). The effect concentrations, EC5 and EC50 increased in the order $GI < OS < MD < CB$ for both *C. reinhardtii* and *Synechocystis* sp.

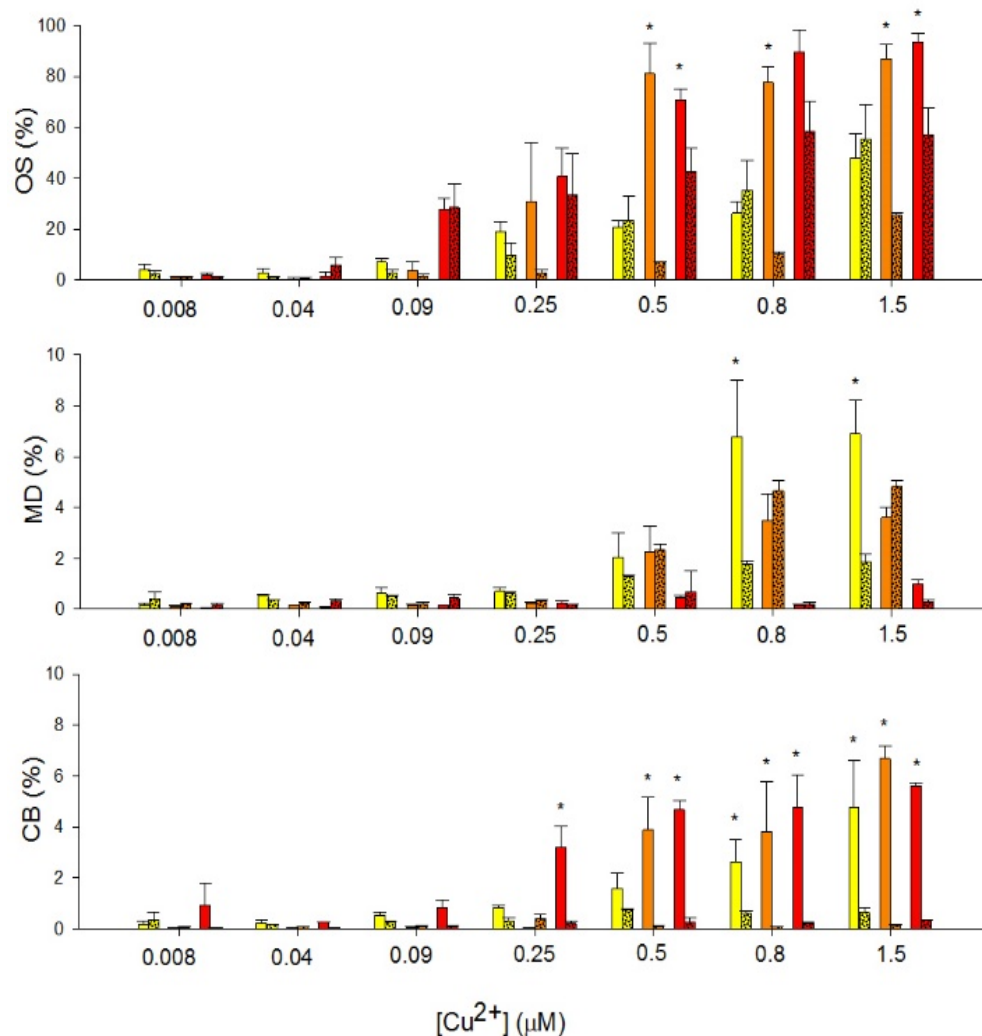


Figure 1. Percentage of affected cells in monoalgal (empty bars) and mixed (dotted bars) *Synechocystis* sp. cultures exposed to increasing Cu concentrations under several visible light treatment conditions. From the top to the bottom oxidative stress (OS), membrane damages (MD) and chlorophyll bleaching (CB). Yellow bars indicate exposure to PAR45, orange bars indicate exposure to PAR 215 and red bars indicate exposure to PAR395. Data are average values $\pm$ standard deviation of three replicates. Exposure time 4h. Asterisks indicate significant differences between monoalgal and mixed cultures tested using Mann-Whitney Rank Sum Test (p<0.05).

The above trend is consistent with the literature showing that growth rate was more sensitive than chlorophyll fluorescence in cyanobacterium *Microcystis aeruginosa* and green alga *Chlorella vulgaris* exposed to comparable copper concentrations (0.08-1.96 μM $[\text{Cu}^{2+}]$) (Hadjoudja et al., 2009). *Synechocystis* sp. was more sensitive to Cu as compared with *C. reinhardtii*. Indeed effects on *Synechocystis* sp. were observed at almost 10 time lower copper concentration than *C. reinhardtii*. The higher sensitivity of *Synechocystis* sp. to copper might be related to several factors such as smaller cell size (Hadjoudja et al., 2009), higher uptake rates (Quigg et al., 2006) or different detoxification mechanisms. However the quantitative relationship between cell surface area and sensitivity to copper is still to establish in microalgae (Levy et al., 2007). *C. reinhardtii* tolerance to copper might be in part linked to the ability of this alga to accumulate copper in specific cellular compartment in order to avoid mismetallation when copper is in excess and other essential metals are limiting (Hong-Hermesdorf et al., 2014). The factors driving such different sensitivity of phytoplankton species to copper are still debated and the issue deserves future studies.

The sensitivity of the two strains to copper decreased significantly under PAR395, as revealed by the increase of EC50s for almost all the tested endpoints (Figures 2 and 3). However, EC50s under PAR215 were comparable with those under PAR45. The changes in EC50 values in *C. reinhardtii* under PAR395 were mainly associated with a shift in the curve slope whereas in *Synechocystis* sp. either changes in the slope and shifts of the EC50 values toward higher copper concentrations were observed. Increase in EC50_{MD} and EC50_{CB} with light intensity were observed for *Synechocystis* sp. exposed to PAR395.

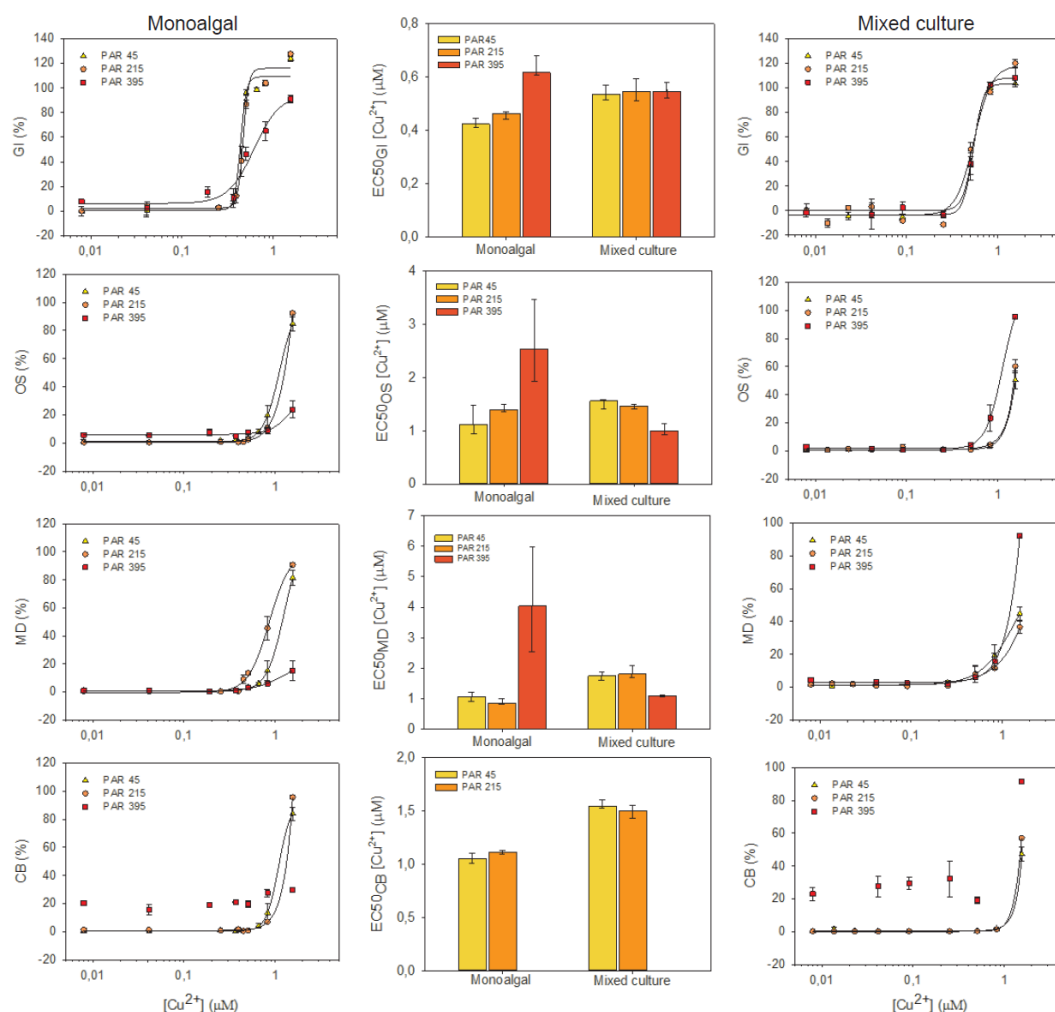


Figure 2. Concentration-response curves for the growth inhibition (GI), oxidative stress (OS), membrane damages (MD) and chlorophyll bleaching (CB) of *Chlamydomonas reinhardtii* exposed to increasing copper concentrations as monoculture (left) and as mixed culture with *Synechocystis* sp. (right) for 48h under different light irradiances (PAR45, PAR215 and PAR395). Data are average values $\pm$ standard deviation of at least three replicates. In the middle panel EC50 values with 95% confidence intervals obtained by fitting the concentration-response curves with a four parameter log logistic model. From the top to the bottom EC50 for growth inhibition (EC50_{GI}), oxidative stress (EC50_{OS}), membrane damages (EC50_{MD}) and chlorophyll bleaching (EC50_{CB}) are expressed as free copper ion concentrations $[Cu^{2+}]$.

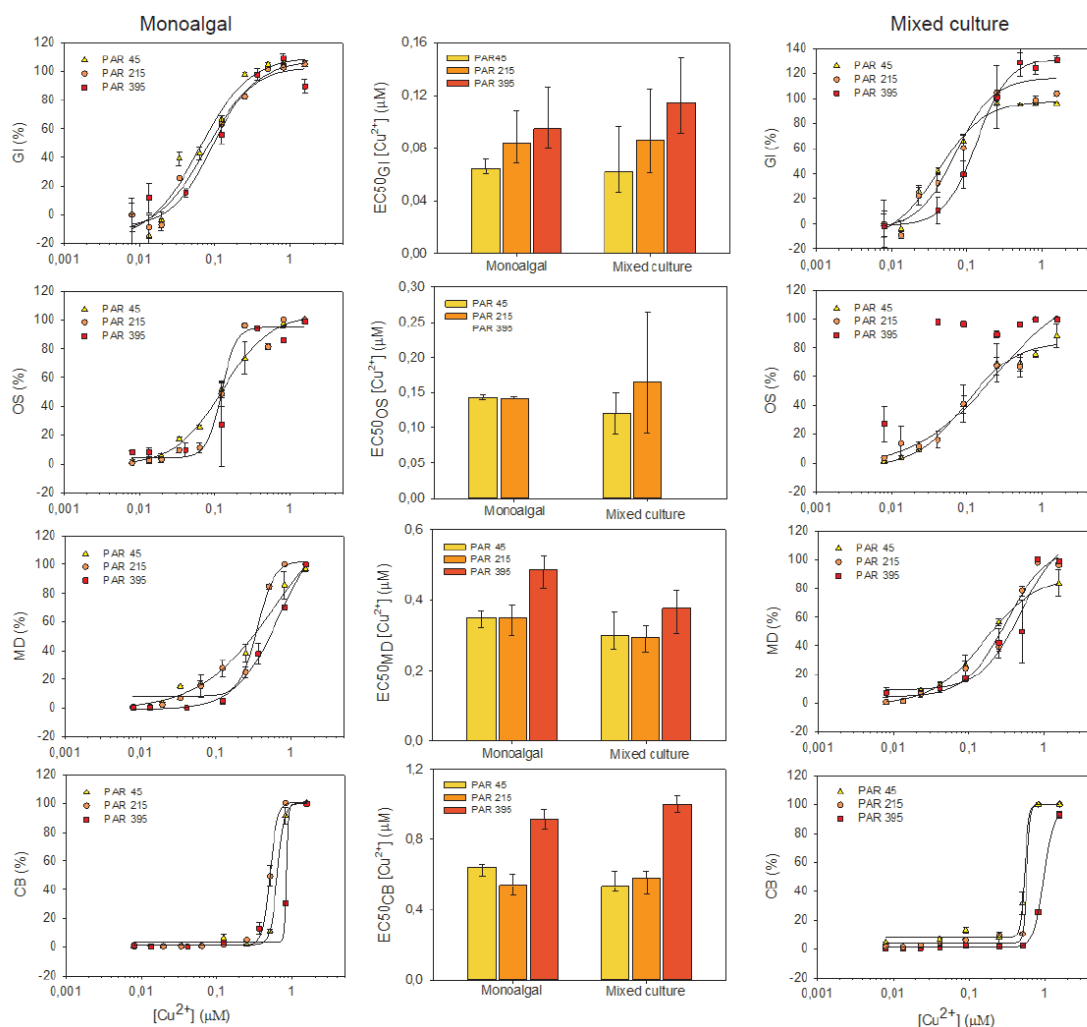


Figure 3. Concentration-response curves for the growth inhibition (GI), oxidative stress (OS), membrane damages (MD) and chlorophyll bleaching (CB) of *Synechocystis* sp. exposed to increasing copper concentrations as monoculture (left) and as mixed culture with *C. reinhardtii* (right) for 48h under different light irradiances (PAR45, PAR215 and PAR 395). Data are average values $\pm$ standard deviation of at least three replicates. In the middle panel EC50 values with 95% confidence intervals obtained by fitting the concentration-response curves with a four parameter log logistic model. From the top to the bottom EC50 for growth inhibition ($EC50_{GI}$), oxidative stress ($EC50_{OS}$), membrane damages ($EC50_{MD}$) and chlorophyll bleaching ($EC50_{CB}$) are expressed as free copper ion concentrations $[Cu^{2+}]$.

However, for *C. reinhardtii* it was not possible to fit the CB concentration-response results since the number of bleached cells exposed to copper changed insignificantly with respect to unexposed controls. For comparison, under the studied conditions, no significant alterations in the algal population traits, such as cell size and pigment autofluorescence, were observed for *Synechocystis* sp during 48h exposure. By contrast, a reduction in chlorophyll fluorescence after 48 h-exposure to PAR395 was found in *C. reinhardtii* cells (Figure S4). The latest observation is consistent with the literature reporting perturbation in chlorophyll biosynthesis under excess of visible light (Li et al., 2009). Similar reduction of chlorophyll content was found in the green alga *Scenedesmus protuberans* exposed to high-intensity light (Ibelings et al., 1994) and might represent a cellular response for reduction of the excess of captured energy under high-intensity light. Similarly to the other biological endpoints, EC50s and EC50os were higher in *C. reinhardtii* cultures incubated under PAR395 than in those incubated under PAR45 and PAR215. Such results agree with the antagonistic effect observed for oxidative stress under combined exposure of *C. reinhardtii* cells to copper and solar simulated light (Cheloni et al., 2014). The antagonistic effect of these two stressors was associated with higher level of expression of genes encoding for antioxidant response enzymes. The EC values corresponding to the OS in *Synechocystis* sp. were comparable for PAR45 and PAR215. The OS results for *Synechocystis* sp. exposed to copper under PAR395 were not fitted with the four parameter log-logistic model due to the elevated standard deviations.

Overall, the long term exposure to high-intensity visible level decrease the toxicity of Cu to both *C. reinhardtii* and *Synechocystis* sp. Indeed, high level of visible light is known to activate several response mechanisms aimed to protect the photosystem, such as the increased

turnover of the protein D1 in the reaction centre of PSII (Li et al., 2009). Such high-intensity light responses could be assumed to repair the damages caused by copper to the photosynthetic system, however further experimental verification is needed. Reactive oxygen species (ROS) production plays a determinant role in metal toxicity to microalgae (Pinto et al., 2003) but also in the cellular response to the excess of light (Li et al., 2009). Oxidative stress is thus a common paradigm for high-intensity light and metal-induced responses. Antioxidant response might thus be effective either to respond to excess of light either to cope with metal toxicity. The present results of reduced Cu toxicity to the green alga and the cyanobacterium under high-intensity visible light, contradicts with the previous literature data revealing enhanced metal toxicity at increasing light irradiation (Singh et al., 2011; Zeng and Wang, 2011). However, in these works the highest tested intensity was $130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, which is in the range of the light levels recommended for OECD and EPA standard toxicity tests (Singh et al., 2011; Zeng and Wang, 2011). To our knowledge this is the first study where the effects of copper on phytoplankton growth, oxidative stress, membrane damages and on chlorophyll bleaching were studied simultaneously under light irradiances above those recommended in standardized toxicity tests and that might be encountered in natural aquatic environments in the upper part of the euphotic zone (Davies - Colley et al., 1984). Therefore the present results could have important implications when extrapolating the data obtained in the laboratory to the natural environment conditions characterized by strongly variable conditions.

3.2 Copper toxicity in mixed cultures under different visible light intensity

To examine the role of specie-specie interactions on copper toxicity under different visible light irradiations the biological responses in mixed cultures were compared with those obtained in monoalgal cultures under the same copper exposure and light intensity conditions. After 4 hour exposure of *Synechocystis* sp. to Cu significant differences were observed in the response of monoalgal and mixed cultures. Lower percentage of cells were affected by oxidative stress and chlorophyll bleaching when *Synechocystis* sp. was co-cultured with *C. reinhardtii* cells (Figure 1). Such results indicate that species-species interaction alters micropollutants toxicity even during a short-term exposure. Surprisingly no changes in copper toxicity were observed during 48h exposure when *Synechocystis* sp. was co-cultured with *C. reinhardtii*. By contrast, the presence of *Synechocystis* sp. altered significantly the sensitivity of *C. reinhardtii* to Cu in a light dependent manner. Under PAR45 and PAR215, *Synechocystis* sp. lead to higher EC50s and decrease of the sensitivity of *C. reinhardtii* to copper. The exact causes for the observed reduced sensitivity of *C. reinhardtii* in mixed cultures is not well understood. However possible changes in the copper speciation and bioavailability due to the production of the exopolymetric substances (EPS) by *Synechocystis* sp. could be anticipated. *Synechocystis* strain (PCC 6803) was shown to produce capsular and soluble EPS involved in cell protection from salt and metal-induced stress (Jittawuttipoka et al., 2013). The release of exudate with metal-complexing properties was suggested to reduce copper sensitivity of *Microcystis aeruginosa* co-cultured with *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Yu et al., 2007).

Copper EC50s of *C. reinhardtii* incubated under PAR395 together with *Synechocystis* sp. were lower than those obtained in monoalgal cultures under the same light irradiation regime, demonstrating that the presence *Synechocystis* sp. increases the sensitivity of *C. reinhardtii* in a combined exposure of Cu and visible light with high intensity. Similar increase in copper sensitivity of *Trachelomonas* sp. in multispecies tests was associated to the production of toxic exudates by *P. subcapitata* (Franklin et al., 2004). The changes in sensitivity observed in the present work might thus be associated to the *Synechocystis* sp. production of allelopathic molecules under copper and high-intensity light stress. Indeed, increased production of cyanobacterial toxins under high light was reported for *Microcystis* strain PCC 7806 (Wiedner et al., 2003), however no evidences for such toxin production in *Synechocystis* sp. PCC6803 exist. Further studies focusing on the biota-mediated changes in the extracellular environment (e.g. release of metal complexing agents by biota, allelopathy etc.) and their implications for the toxicity of naturally occurring or synthetic chemicals towards phytoplankton communities under multiple stress conditions would be necessary to have further insights on the specie-species interactions and their consequences for contaminants toxicity.

Overall, these results revealed that *C. reinhardtii*-*Synechocystis* sp. interactions might be beneficial for *Synechocystis* sp. at short-term exposure. They could be beneficial for *C. reinhardtii* at long-term exposure under low-intensity light, however might become detrimental under high-intensity visible light. Specie-specie interactions are important players in phytoplankton dynamics, therefore ecotoxicity studies considering such biotic interactions are essential for reduction of the uncertainties associated with standard single-specie tests.

4 Conclusions

Specie-specie interaction influenced copper toxicity dissimilarly depending on the exposure time and light conditions. At low and medium-intensity light, the presence of *Synechocystis* sp. has reduced the sensitivity of *C. reinhardtii* to Cu, whereas at high-intensity light the opposite effect was observed. The influence of *C. reinhardtii* on the responses of *Synechocystis* sp. to copper was found only at short incubation time. For a given light condition, *Synechocystis* sp. was more sensitive to copper than *C. reinhardtii* in single specie tests. Among the tested biological responses, the growth inhibition was the most sensitive end point, followed by the oxidative stress, membrane damage and chlorophyll bleaching in both single- and multi-specie tests. Long-term exposure to copper under visible light of increasing intensity reduced the sensitivity of copper for both phytoplankton. The above results have important environmental implications for the extrapolation of the data obtained from standard toxicity test to the natural environment where the aquatic microorganisms experience multiple stressor influence.

Supplemental Data

The Supplemental Data are available at DOI:

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Data availability

The data are available on request to the corresponding author.

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