The role of antioxidant response and nonphotochemical quenching of chlorophyll fluorescence in long-term adaptation to Cu-induced stress in Chlamydomonas reinhardtii

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Abstract

The aim of present study was to analyse selected aspects of the mechanism of protection of the photosynthetic apparatus and antioxidant activity in response to excessive copper concentrations in wall-less strains of *Chlamydomonas reinhardtii* not adapted and adapted for growth in the presence of elevated copper level. The measured parameters were photosynthetic pigment content, prenyllipid antioxidant (α-tocopherol, plastoquinone pool) content, peroxidase activity, and nonphotochemical quenching efficiency. The results obtained suggest that the increased content of tocopherol and plastoquinone, as well as the increased efficiency of nonphotochemical quenching of chlorophyll fluorescence, play a role in the acquisition of tolerance to copper. The role of light in the enhancement of copper toxicity and the role of POX in response to elevated copper have also been shown.

1. Introduction

Copper is a crucial micronutrient for photosynthetic organisms. The electrochemical potential of Cu²⁺/Cu⁺ is -268 mV and fits in the physiological range, which makes this element useful for participation in the catalysis of redox reactions occurring in cells (Nies 1999). Copper is a prosthetic group in many enzymes including cytochrome oxidase and Cu/Zn superoxide dismutase; it also occurs in protein electron carriers, such as plastocyanin. This makes copper essential for the functioning of photosynthesis, respiration, and many other metabolic processes (Nagajyoti et al. 2010). However, in supraoptimal concentrations, copper is highly toxic. Photosynthetic organisms are especially sensitive to excessive copper, since the disturbance of metabolism occurs when the intracellular copper content is only slightly higher than the optimal level. Due to the fact that copper ions are more mobile in the water than in the soil, this heavy metal poses a serious threat to algae and aquatic plants (Fernandes and Henriques 1991). Freshwater ecosystems are particularly threatened by copper contamination resulting from anthropogenic activities, such as mining, smelting, chemical industry, and many others (Nagajyoti et al. 2010; Andresen and Küpper 2013).

The harmful effects of heavy metals on living organisms are pleiotropic (Nagajyoti et al. 2010). Taking into account copper-induced toxicity, there are two major mechanisms. A prime target of copper toxicity is the light phase of photosynthesis (Küpper and Andresen 2016). This element is known to interact with Tyr₂ and Tyr₃, non-heme Fe, cyt b₅₅₉ and sites close to pheophytin, Qₐ, and Qₐ binding pockets in photosystem II (PS II), which leads to inhibition of O₂ evolution (Burda et al. 2003; Yruela 2005; DalCorso 2012). Copper is also able to substitute Mg²⁺ in Chlorophyll (Chl) which leads to excitation energy loss (Küpper and Andresen 2016). Apart from its inhibitory action on light reactions, copper is also known to inhibit enzymes crucial for the dark phase, such as Rubisco and phosphoenolpyruvate carboxylase, and to damage the chloroplast structure (DalCorso 2012). The second important mechanism of toxicity is connected with the redox properties of copper, which allows this element to undergo unwanted and uncontrolled redox cycling in living cells. These reactions lead to the formation of reactive oxygen species (ROS); in particular, the reaction of Cu⁺ with H₂O₂ results in the formation of the most dangerous ROS, the
hydroxyl radical. Due to this aspect of copper chemistry, this element is included in the redox-active heavy metal group (DalCorso 2012; Stoiber et al. 2013).

Oxidative stress, which is a situation of excessive ROS production in cells, can be induced by the direct action of copper ions, but also indirectly, as a result of the disturbance of photosynthesis and other metabolic processes (Pinto et al. 2003). Inhibition or slowing of photosynthetic electron transfer leads to overexcitation of the photosynthetic apparatus, resulting in unwanted side reactions leading to damage to pigments and proteins, as well as to the formation of singlet oxygen ($^1O_2$) and superoxide (O$_2^-$•−) (Edreva 2005). Therefore, protective mechanisms, such as the thermal dissipation of absorbed light energy, may play a role in alleviating copper toxicity. Nonphotochemical quenching of chlorophyll fluorescence is therefore a mechanism preventing ROS formation in cells (Finazzi et al. 2006). To cope with ROS already formed, living organisms evolved robust ROS-detoxifying systems based on low-molecular-weight antioxidants and antioxidant enzymes (Pinto et al. 2003). As the major source of ROS in photosynthetic organisms are chloroplasts, plastidial antioxidants are very important for antioxidant defence in algae. This group includes hydrophilic compounds, such as ascorbate (Asc) and glutathione (GSH), as well as hydrophobic antioxidants belonging to prenyllipids. The latter group, comprising carotenoids (Car), tocopherols (Toc), and plastoquinol (PQH$_2$), is very important for the protection of thylakoid membranes (Nowicka et al. 2021a). Superoxide dismutases, catalase, ascorbate peroxidase, and other peroxidases are examples of ROS-detoxifying enzymes (Gechev et al. 2006). Increased content of low-molecular-weight antioxidants and antioxidant enzyme activities are considered important for the acquisition of heavy metal tolerance (Nowicka et al. 2016a; Nowicka 2022).

The green microalga *Chlamydomonas reinhardtii* P.A. Dangeard is a model photosynthetic microorganism widely used in the research concerning heavy metal toxicity and tolerance (Hanikenne 2003). This microalga is easy to grow, metabolically profiled, and its genome has been sequenced; all of this makes it useful in experiments (Nowicka et al. 2016a). The short life cycle, the haploid vegetative stage, and the ability to adapt to various stress conditions are characteristics that make *C. reinhardtii* a good model organism for research concerning microevolutionary processes (Hanikenne 2003; Pluciński et al. 2018).

Pluciński and co-workers (Pluciński et al. 2018, 2021) starting from cell wall-less *C. reinhardtii* strain CW15 have obtained copper-tolerant strains, which are a subject of further investigation. The aim of the present study was to examine the role of prenyllipid antioxidants, peroxidase activity, and non-photochemical quenching of chlorophyll fluorescence in the acquisition of tolerance to copper toxicity. The content of photosynthetic pigments, Toc, PQH$_2$, plastoquinone (PQ), peroxidase POX) activity, as well as the efficiency of nonphotochemical quenching of chlorophyll fluorescence were measured in copper-tolerant and nontolerant paternal strains exposed to elevated copper concentrations.

### 2. Materials And Methods
2.1. Strains used, growth conditions, and photosynthetic pigments determination

*Chlamydomonas reinhardtii* was cultured aseptically in Erlenmeyer flasks (250 ml) in Sager-Granick (SG) medium supplemented with 100 mM mannitol, 7.5 mM sodium acetate and 1.7 mM citrate, on a shaker, in a growth chamber at 22 ± 2°C as described in (Pluciński et al. 2018). The parental strain (“N1”) and cell wall-containing population 11-32b (“Wall”) were grown at a Cu²⁺ concentration of 0.25 µM nominal for SG medium, the “Cu2” strain was grown on modified SG medium with Cu²⁺ concentration elevated to 5.25 µM for more than a year. The “Cu200” population was obtained from the Cu2 strain as a result of culture in the presence of 200 µM Cu²⁺ for more than a year. Algal cultures from the three experimental populations were weekly inoculated into fresh medium (3 ml of 1-week-old culture per 100 ml of new medium). The algae were cultivated under the 16:8 light:dark cycle at 50 µmol m⁻² s⁻¹ photosynthetic active radiation from a fluorescent lamp.

In the experiment aimed at the evaluation of photosynthetic pigments and POX activity, algae were cultured on a 48-well plate. The initial amount of chlorophyll was the same in all variants. The N1 and Cu2 strains were grown either in normal (50 µmol m⁻² s⁻¹) or shade (10 µmol m⁻² s⁻¹) light on the media containing either 0.25 or 50 µM Cu²⁺ in six biological repetitions.

For Chl *a*, Chl *b* and total Car measurements, 200 µl of cell suspensions were centrifuged (5 min, 9000 g). The obtained pellet was extracted with acetone, extract was centrifuged again (5 min, 9000 g) to remove cell debris, and the photosynthetic pigment content was determined spectrophotometrically according to (Lichtenthaler 1987).

In the experiment aimed at the evaluation of prenyllipid antioxidants, N1, Cu2 and Cu200 strains were grown in Erlenmeyer flasks (250 ml), in control medium containing 0.25 µM Cu²⁺ and in the media containing 25, 50, 100 and 200 µM Cu²⁺ in four repetitions. The initial amount of chlorophyll was the same in all variants. The samples (10 ml per each) were taken after 7 days of culture growth, and centrifuged (5 min, 5400 g, 4°C). The obtained pellet was frozen in liquid nitrogen and then extracted as described in Section 2.3.

In the experiment aimed at the evaluation of chlorophyll fluorescence parameters, the N1 and Cu2 strains were grown in normal light (50 µmol m⁻² s⁻¹ light) on the media containing either 0.25 or 50 µM Cu²⁺ in 8 repetitions, in 48-well plate. The initial amount of chlorophyll was the same in all variants. Four preincubation variants were applied: 2 h under 50 µmol m⁻² s⁻¹ light or 2 h in darkness; in the presence or absence of a solution with organic carbon and phosphorous source. The solution used contained sodium acetate, sodium citrate, K₂HPO₄ and KH₂PO₄. The added solution (20 µl for 1 ml of culture) induced an increase in the concentrations of listed components in the medium by 80%. After preincubation, plates were dark-adapted for 20 min and chlorophyll fluorescence parameters were measured.

2.2. Peroxidase activity determination
Peroxidase activity was determined using a classical colorimetric assay with pyrogallol (Maehly and Chance 1954). Samples (2 ml of cultures) were centrifuged (3 min, 12000 g), resuspended in 250 µl of 100 mM phosphate buffer pH 6.0, then freeze-thawed / thawed three times in liquid nitrogen to disrupt the cells. The obtained suspension was centrifuged again (5 min, 12000 g) to remove cell debris. The following procedure has been applied: 200 µl of extract was added to the reaction mixture (500 µl of distilled water + 100 µl of 100 mM phosphate buffer pH 6.0 + 100 µl of 0.5% pyrogallol solution in 100 mM phosphate buffer pH 6.0), then 100 µl of 3% H$_2$O$_2$ was added. The whole mixture was mixed by pipetting and the increase in purpurogallin concentration was monitored by absorbance detection at $\lambda = 420$ nm at 20°C.

POX activity was expressed as a change in absorbance for a second, normalized to the chlorophyll content, and multiplied by 1000.

2.3. Prenyllipid determination

The extraction of prenyllipids and determination of $\alpha$-Toc, PQH$_2$ and PQ using RP-HPLC were performed as described in (Nowicka and Kruk 2012). To avoid oxidation of PQH$_2$ during acetone extraction, the sample was not incubated with the solvent for a time longer than 2 min, then centrifuged (5 min, 9000 g), evaporated in a stream of nitrogen, dissolved in 200 µl of methanol, and injected into an HPLC system. HPLC analysis of prenyllipids was performed in the following system: C$_{18}$ reverse-phase column (Teknokroma, Spain, 5 µm, 25 cm × 0.4 cm), eluent - methanol:hexane (340:20, v/v), flow rate of 1.5 ml/min, absorption detection at $\lambda = 255$ nm, fluorescence detection at $\lambda_{ex} = 290$ nm, $\lambda_{em} = 330$ nm. The concentration of prenyllipids in the extracts was evaluated by comparing with the respective standards and normalized on the basis of the total Chl content (mol/100 mol chlorophyll $a + b$).

2.4. Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters were measured using Open FluorCam FC 800-O (Photon Systems Instruments, Brno, Czech Republic) as described in (Nowicka et al. 2016a). Weak red modulated light was applied as measuring light, white light of an intensity of 2600 µmol photons m$^{-2}$ s$^{-1}$ was used as saturating light (pulse duration 1000 ms). Red light of an intensity of 220 µmol photons m$^{-2}$ s$^{-1}$ was used as actinic light. The measured parameter was nonphotochemical quenching (NPQ) of chlorophyll fluorescence, calculated as $(F_m - F_{m'})/F_{m'}$ ($F_m$, maximum fluorescence; $F_{m'}$, maximum fluorescence in sample exposed to actinic light) (Maxwell and Johnson 2000). Induction of NPQ was measured during 30 min of illumination with actinic light, then NPQ relaxation was monitored in darkness for 25 min. Saturating pulses were applied as indicated in Fig. 3.

2.5. Statistical analyses

Factors affecting peroxidase activity, chlorophyll $a$, chlorophyll $b$, and carotenoids concentrations were first analysed by full-factorial linear models with line (N1 and Cu2), copper exposure and light exposure, and all their interactions as fixed factors. Models were then reduced by stepwise removal of
nonsignificant interactions. In the reduced models, the effect of light was always significant. Finally, the analyses were carried out separately for each of the two light conditions.

Factors affecting concentrations of α-tocopherol (α-Toc), PQH$_2$ and PQ, total PQ (PQ + PQH$_2$) and the ratio of PQ to total PQ were first analysed using linear models with line (Wall, N1, Cu2, and Cu200) introduced as categorical factor and copper concentration as linear and quadratic predictor. To further explore the interaction of interest, i.e. the interaction between line and copper concentration, the analyses were carried out separately for each line.

Changes in PQ concentration over time were measured in Cu2 and N1 lines in light or darkness and in the absence or presence of copper (0.25 and 50 µM copper respectively). The curve of changes in plastoquinone over time was non-linear. For each combination of those factors, we picked up the highest concentration and visualised it.

NPQ induction values in Cu2 and N1 lines were first plotted against the time of incubation separately for cultures without and with carbon, without and with exposure to copper and in light and darkness pretreatments. The highest value for each curve was then analysed separately for the two carbon conditions. Both analyses were full-factorial linear models with line (N1 and Cu2), copper exposure and light pretreatment, and all their interactions introduced as fixed factors.

The analyses were carried out in R-4.0.5 (R Core Team 2022), with lm() function, which is generic to R. Tables with results were generated with the Anova() function with the Type III sum of squares from the car package (Fox and Weisberg 2019). Figures were prepared in the ggplot2 package (Wickham 2016).

2.6. Data availability

The data sets and the R scripts used are stored in the Open Science Framework repository (https://osf.io/gyq42/?view_only=6f556d7526394f7f808fa69be97d251f).

3. Results

3.1. Photosynthetic pigments

The levels of Chlorophyll $a$, Chlorophyll $b$ and total carotenoids were higher in Cu2 line cultures than in N1 line cultures. But the presence or absence of elevated copper and the two light conditions affected the degree of those differences.

Chlorophyll $a$ concentration was shaped by all the main factors in the model and also the interactions of line × copper exposure, line × light and copper × light (Table 1). Chlorophyll $a$ was clearly higher in the Cu2 line compared to the N1 line and in both lines was lower in samples exposed to copper. The analyses carried out separately for light and shade conditions revealed that the effects of copper exposure were stronger in light than in shade (Table 2). In all but one combination of factors, exposure to copper reduced chlorophyll $a$ content compared to treatment without copper. The exception was the Cu2 line measured in
the shade, where the chlorophyll \( a \) concentration was not affected by the addition of copper (Fig. 1a). In the N1 strain, exposure to stronger light led to an increase in chlorophyll \( a \) content both in the presence and absence of excessive copper; however, for copper-exposed algae this effect was less pronounced. For Cu2, a slight increase was observed for the control culture, whereas a decrease in chlorophyll \( a \) content was observed in cultures exposed to copper illuminated with stronger light, compared to series grown in shade (Fig. 1a).

### Table 1

Results of the linear model in which the activity of peroxidase (POX), chlorophyll \( a \) (Chl \( a \)), chlorophyll \( b \) (Chl \( b \)), and carotenoids (Car) concentrations were analysed with respect to the experimental line, copper exposure and light exposure, and significant interactions of those factors. \( df \) – degrees of freedom; \( F \) – calculated statistics; \( p \) – probability of obtaining a result by chance.

<table>
<thead>
<tr>
<th>Factor</th>
<th>POX activity</th>
<th>Chl ( a )</th>
<th>Chl ( b )</th>
<th>Car</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>p</td>
<td>df</td>
</tr>
<tr>
<td>line</td>
<td>1</td>
<td>9.6</td>
<td>0.003</td>
<td>1</td>
</tr>
<tr>
<td>copper</td>
<td>1</td>
<td>6.1</td>
<td>0.017</td>
<td>1</td>
</tr>
<tr>
<td>light</td>
<td>1</td>
<td>8.9</td>
<td>0.004</td>
<td>1</td>
</tr>
<tr>
<td>line×copper</td>
<td>1</td>
<td>7.9</td>
<td>0.007</td>
<td>1</td>
</tr>
<tr>
<td>line×light</td>
<td>1</td>
<td>5.4</td>
<td>0.024</td>
<td>1</td>
</tr>
<tr>
<td>copper×light</td>
<td>1</td>
<td>7.6</td>
<td>0.009</td>
<td>1</td>
</tr>
<tr>
<td>Residuals</td>
<td>43</td>
<td></td>
<td></td>
<td>41</td>
</tr>
</tbody>
</table>
The chlorophyll \( \textit{b} \) concentration showed a pattern similar to that of chlorophyll \( \textit{a} \) (Table 1), especially in terms of the weaker effects of the line and the exposure to copper in shade compared to light conditions (Table 2) and the lack of decline of chlorophyll \( \textit{b} \) in the Cu2 line exposed to copper and measured in shade (Fig. 1b).

The concentration of carotenoids was shaped by all main factors in the model and also the interactions of line × copper exposure, line × light and copper × light (Table 1). It was clearly higher in Cu2 compared to the N1 line and lower in samples exposed to copper. Analyses carried out separately for light and shade conditions revealed that in each of those conditions, the effect of line and the effect of copper exposure was visible, but those two factors did not interact (Table 2, Fig. 1c). The response of carotenoids to light conditions was similar to that observed for chlorophylls (Fig. 1c).

### 3.2. Peroxidase activity

POX activity was affected by all main factors in the model and the interaction between line and copper exposure (Table 1, Fig. 1d). In short, it was on average higher in Cu2 compared to the N1 line, in samples exposed to 50 \( \mu \text{M} \) \( \text{Cu}^{2+} \) compared to control, and in light conditions compared to shade. Analyses carried out separately for light and shade revealed that it was only under light conditions, where the effects of line and copper were pronounced and the highest value of POX activity was in the Cu2 line exposed to 50 \( \mu \text{M} \) \( \text{Cu}^{2+} \) (Table 2a). In shade, none of the factors differentiated peroxidase activity (Table 2b).
3.3. Prenyllipid content

Concentration of α-Toc was shaped by the significant interaction between the line and the copper concentration (Table 3, Fig. 2a). The interaction steamed from the fact that the four lines responded differently to copper. Specifically, the Cu200 line, which on average had the highest level of α-Toc, showed a quadratic response to increasing copper concentration and exhibited the highest α-Toc level for 100 µM Cu$^{2+}$. The Cu2 line exhibited increasing α-Toc levels for lower copper concentration and a marked decrease for 200 µM Cu$^{2+}$, but the quadratic relationship with copper concentration was only marginally significant (Table 4). Lines N1 and Wall showed generally low α-Toc concentration. The Wall line exhibited a quadratic effect of copper concentration, but was much less pronounced than the other two lines. Similarly to Cu2, there was a decrease in α-Toc content for the highest copper concentration applied (Fig. 2a). The N1 line was insensitive to copper concentration (Table 4).

Table 3
Results of linear model in which α-tocopherol (α-Toc), plastoquinol (PQH$_2$) and plastoquinone (PQ), total PQ (PQ$_{tot}$ = PQH$_2$ + PQ) and PQ/PQ$_{tot}$ ratio were analyzed by linear models with line, copper concentration introduced as linear and quadratic term and their interaction.

<table>
<thead>
<tr>
<th>Factor</th>
<th>α-Toc</th>
<th>PQH$_2$</th>
<th>PQ</th>
<th>PQ$_{tot}$</th>
<th>PQ / PQ$_{tot}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>line</td>
<td>3</td>
<td>34.9</td>
<td>&lt; 0.001</td>
<td>36.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>copper</td>
<td>1</td>
<td>5.7</td>
<td>0.019</td>
<td>3.2</td>
<td>0.080</td>
</tr>
<tr>
<td>copper$^2$</td>
<td>1</td>
<td>9.1</td>
<td>0.003</td>
<td>7.1</td>
<td>0.009</td>
</tr>
<tr>
<td>line×copper</td>
<td>3</td>
<td>5.7</td>
<td>0.002</td>
<td>6.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Error</td>
<td>66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4
Results of regression analyses $\alpha$-tocopherol ($\alpha$-Toc), plastoquinol (PQH$_2$) and plastoquinone (PQ), total PQ (PQ$_{tot} = $ PQH$_2$ + PQ) and the PQ / PQ$_{tot}$ ratio with respect to copper concentration, separately for each line. The quadratic term was removed from the model if its p > 0.1.

<table>
<thead>
<tr>
<th></th>
<th>copper</th>
<th></th>
<th>copper$^2$</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
<td>p</td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td>$\alpha$-Toc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wall</td>
<td>3.06</td>
<td>1, 12</td>
<td>0.106</td>
<td><strong>6.84</strong></td>
<td>1, 12</td>
</tr>
<tr>
<td>N1</td>
<td>2.47</td>
<td>1, 18</td>
<td>0.134</td>
<td></td>
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</tr>
<tr>
<td>Cu2</td>
<td><strong>7.86</strong></td>
<td>1, 17</td>
<td><strong>0.0122</strong></td>
<td>3.62</td>
<td>1, 17</td>
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<tr>
<td>Cu200</td>
<td>2.9</td>
<td>1, 17</td>
<td>0.105</td>
<td><strong>6.52</strong></td>
<td>1, 17</td>
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<tr>
<td>PQH$_2$</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Wall</td>
<td>58.8</td>
<td>1, 12</td>
<td>&lt; 0.0001</td>
<td><strong>9.4</strong></td>
<td>1, 12</td>
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<tr>
<td>N1</td>
<td>11.5</td>
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<td>0.086</td>
<td><strong>5.27</strong></td>
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<td>PQ</td>
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<td>Wall</td>
<td><strong>81.8</strong></td>
<td>1, 13</td>
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<tr>
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<td>1, 17</td>
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<td>PQ$_{tot}$</td>
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<td>Wall</td>
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<td>1, 13</td>
<td>0.518</td>
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<td>1, 17</td>
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<td>1, 17</td>
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<td>PQ / PQ$_{tot}$</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Wall</td>
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<td>1, 12</td>
<td>&lt; 0.0001</td>
<td><strong>6.99</strong></td>
<td>1, 12</td>
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<td>N1</td>
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<td><strong>13.1</strong></td>
<td>1, 17</td>
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</tbody>
</table>
The total PQ level showed the highest values for the Cu2 and Cu200 lines, while it was lower for the Wall and N1 lines (Fig. 2b). A quadratic relationship with copper concentration was visible in all lines (Fig. 2b), but significant in the case of Cu200 and N1 lines (Table 4). The total PQ content did not change significantly when the Wall line was exposed to excessive copper. The N1 and Cu2 strains showed an increase in total PQ for lower copper concentrations applied and then a decrease. For N1 strain, this decrease was observed for 100 and 200 µM Cu$^{2+}$, whereas for Cu2 strain only for 200 µM Cu$^{2+}$. An increase in total PQ level was observed in Cu200 strain exposed to copper for all copper concentrations tested compared to the control series.

The PQH$_2$ level showed a similar pattern to that of α-Toc (Table 3, Fig. 2c). In lines Cu200 and Cu2, the PQH$_2$ concentration had high values, which were affected by the copper concentration in a non-linear manner, with levels initially increasing with the increase in the copper concentration and decreasing for the 200 µM Cu$^{2+}$. In the case of the Cu2 line, this decrease was very pronounced, nearly to the levels observed for 200 µM Cu$^{2+}$ in other two lines, yet the quadratic term showed only marginal significance (Table 4). Wall line showed a quadratic reaction to copper concentration, and the N1 line had hardly no PQH$_2$ except for the 0.25 µM Cu$^{2+}$. Exposure to elevated copper caused the oxidation of almost all PQH$_2$ in the N1 strain for all the Cu concentrations applied and in the Wall and Cu2 strains for the highest copper concentration applied.

The PQ content exhibited the highest values for the N1 line, but with a quadratic relationship with the copper concentration (Fig. 2d). In the case of the Wall line, PQ had intermediate levels and it actually increased with increasing copper concentration. The Cu2 line had low levels of PQ at low copper concentration, but those levels increased for 100 and 200 µM Cu$^{2+}$. No such increase was observed in the Cu200 line, which maintained the lowest levels of PQ and was not affected by the copper concentration (Table 4).

The PQ to total PQ ratio differed greatly between the lines and also in how the lines reacted to copper levels, as indicated by the significant interaction between the line and copper (Table 3, Fig. 2e). For the N1 strain PQ/PQ$_{tot}$ reached the values of 1 for all copper concentration except the 0.25 µM Cu$^{2+}$. The share of PQ in the total pool of plastoquinone (PQH$_2$ + PQ) of the Wall line increased exponentially, starting at intermediate values. Increasing copper concentration caused PQ/PQ$_{tot}$ to increase exponentially in the Cu2 line, but not in the Cu200 line.

### 3.4. Photosynthetic parameters
The copper-adapted line, Cu2, displayed enhanced efficiency of NPQ induction compared to the N1 line, both in the copper-exposed and control cultures, independently of light pretreatment and carbon exposure (Fig. 3). Induction of NPQ in the Cu2 line was usually faster than in the N1 line (Fig. 3). Statistical analyses of the maximum NPQ values showed that all factors and their interactions were influential (Table 5). Specifically, NPQ induced in dark pretreated cultures was more efficient than NPQ induced in light pretreated cultures, however, in the Cu2 line the effect of darkness was more pronounced than in N1. This trend was observed in both control and copper-exposed algae. Exposure to copper led to a decrease in NPQ in Cu2, while copper-induced stress in the N1 line generally led to a slight enhancement of NPQ efficiency (Table 5, Fig. 3). Additional nutrients induce an increase in the differences in NPQ between measured strains (Fig. 3).

### Table 5

Results of the linear model in which the maximum value of the NPQ efficiency was analysed with respect to line, copper exposure, and light or dark pretreatment and their interactions. Analyses were run separately for a) cultures without an additional amount of organic carbon and b) cultures with addition of organic carbon.

<table>
<thead>
<tr>
<th></th>
<th>a) without carbon</th>
<th>b) with carbon</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>F</td>
<td>df</td>
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<tr>
<td>line</td>
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<tr>
<td>light</td>
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<tr>
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<tr>
<td>line×light×copper</td>
<td>14.27</td>
<td>1, 24</td>
</tr>
</tbody>
</table>

### 4. Discussion

#### 4.1. Photosynthetic pigments

The results of the measurements of photosynthetic pigment content show that light enhances the toxic effect of copper. In particular, this may be concluded on the basis of the observed decrease in the pigment level in Cu2 exposed to copper grown in normal light, when compared to the respective series cultured in shade (Fig. 1). The enhancement of the toxic effect of copper by light has been described in the literature (Lu and Zhang 1999; Knauert and Knauer 2008; Nielsen and Nielsen 2010).

#### 4.2. Role of peroxidase
The importance of antioxidant enzymes for acclimation to heavy metal-induced stress has been widely documented (Mourato et al. 2012; Sytar et al. 2013; Nowicka 2022). Increased activities of SOD, CAT and APX were observed in copper-exposed *C. reinhardtii* (Zheng et al. 2011; Jiang et al. 2016; Nowicka et al. 2021b). Taking into account POX activity, it was enhanced in the copper-exposed green algae *C. reinhardtii, Scenedesmus acuminatus, Chlorella vulgaris*, and in the diatom *Odontella mobiliensis* (Manimaran et al. 2012; El-Naggar and Sheikh 2014; Jiang et al. 2016; Hamed et al. 2017).

The observed increase in POX activity in copper-exposed N1 and Cu2 strains (Fig. 1d) confirms the role of POX in response to copper-induced stress. The fact that this effect is more pronounced in algae grown in higher light intensity supports the hypothesis regarding the enhancement of copper-toxicity by light. The impact of light conditions on POX activity suggests that this enzyme plays a role in protecting *C. reinhardtii* from ROS formed during photosynthesis. The usually observed increased activity of POX in the Cu2 line compared to N1 may suggest that the increase in the activity of this enzyme plays a role in long term acclimation.

### 4.3. Role of the prenyllipids

Acclimation to heavy metal-induced stress is often accompanied by an increase in the antioxidant content in the exposed organism. Usually, the degree of this increase is proportional to the applied concentration of heavy metal salt. The application of concentrations high enough to cause severe stress results in the depletion of antioxidants (Elbaz et al. 2010; Nowicka et al. 2016b, 2020).

The increase in α-Toc level in response to copper was observed in both higher plants and algae (Zengin and Munzuroglu 2005; Luis et al. 2006; Collin et al. 2008; Nowicka et al. 2016a; Hamed et al. 2017). Experiments on *C. reinhardtii* showed that usually the increase in α-Toc content occurs for lower copper concentrations applied, whereas for higher ones α-Toc level decreases, most probably due to enhanced oxidative degradation of this compound (Luis et al. 2006; Nowicka et al. 2016a). The increase in the PQH₂ content and the entire PQ pool was observed in *C. reinhardtii* exposed to chromium and cadmium salts (Nowicka et al. 2016a, 2020). On the other hand, the strain *C. reinhardtii* 11-30b (called Wall strain in the present paper) exposed to copper concentrations high enough to significantly inhibit Chl synthesis showed an increased PQ/PQₜₒₜ ratio, which probably resulted from the enhanced oxidation of PQH₂ due to ROS scavenging or inhibition of PQ reduction in PS II, or both of these mechanisms (Nowicka et al. 2016a).

The increased α-Toc, PQH₂ and PQₜₒₜ content observed in copper-adapted strains (Fig. 2., Table 3) suggests that the accumulation of these prenyllipid antioxidants may be an important way to provide greater tolerance to copper. It is also worth mentioning that when the Cu200 strain, adapted to the medium containing 200 µM Cu²⁺, was grown in lower copper concentrations, the prenyllipid content decreased, which may be a result of the decreased demand for these compounds.

The decrease in α-Toc and PQₜₒₜ, as well as the increased share of PQ in the total PQ pool in the Wall, N1, and Cu2 strains exposed to copper (Fig. 2, Table 4) is an indicator of the occurrence of increased copper-
induced oxidative stress. The fact that Cu2 strain shows such an effect in the presence of 200 µM Cu2+, in spite of containing “basal” α-Toc and PQtot levels similar to Cu200, shows that the increased content of these compounds is not the only mechanism responsible for the enhancement of copper tolerance. The fact that the decrease in prenyllipids and enhanced oxidation of PQH2 in the N1 strain occurs in the presence of Cu2+ concentration in which there is no such effect for Wall strain, supports the hypothesis (already proposed in the literature) that the complexation of heavy metal ions by the cell wall is an important protective mechanism (Macfie et al. 1994; Prasad et al. 1998; Macfie and Welbourn 2000).

4.4. Role of NPQ

Copper dose-dependent enhancement of NPQ efficiency was observed in C. reinhardtii after two weeks of exposure (Nowicka et al. 2016a). The increase in the qN parameter, also referring to the nonphotochemical quenching capacity of Chl fluorescence, has been also observed in C. reinhardtii after 96 h of copper-exposure (Juneau et al. 2002). The faster and more pronounced NPQ observed in the Cu2 strain (Fig. 3) suggests that NPQ plays a role in the adaptation to elevated copper concentrations. As the observed difference concerns the fast-relaxing NPQ component, the enhanced NPQ most probably is the result of a more efficient qE. In C. reinhardtii, this mechanism strongly depends on the amount of protective protein LHCSR3 (Bonente et al. 2011). Interestingly, unlike higher plants, in C. reinhardtii the xanthophyll cycle pigments are less important for qE. However, their content increases in algae acclimated to growth in higher light intensity (Quaas et al. 2015). The increase in NPQ observed in dark-preincubated algae compared to light-preincubated ones may result from the fact that light-preincubated C. reinhardtii has its metabolism “tuned into” photosynthesis, enabling fast consumption of light phase products (Fig. 3) This would result in lower ΔpH gradient across thylakoid membranes, compared to actinic light-exposed dark-preincubated algae; and one needs to remember about the regulatory role of ΔpH gradient in qE (Bonente et al. 2011).

The presence of organic carbon has a positive impact on algae growth in spite of down-regulation of photosynthetic efficiency under conditions of mixotrophic growth (Johnson and Alric 2012). An additional energy source modulates fatty acid metabolism and glycolysis and increases the share of cyclic electron transport in the reactions of the light phase of photosynthesis (Chapman et al. 2015). The addition of acetate to the medium induces an increase in the NPQ due to ΔpH-dependent down-regulation of PSII and the transition from state 1 to state 2 (Endo and Asada 1996). The first of the mentioned mechanisms is similar to that occurring during high light exposure (Tian et al. 2019). The enhanced activity of cyclic electron transport is associated with state 1 to 2 transitions (Finazzi 2005), and was shown to be the form of protection of PSII from the negative impact of Cd2+ (Wang et al. 2013). Algae grown in the presence of acetate are less susceptible to photoinhibition (Roach et al. 2013).

In the N1 line, preincubation in light and in the presence of acetate had a negative impact on the increase in maximum NPQ in algae exposed to copper (Fig. 3, Table 5). This may be an effect of the protective role of organic carbon present in the medium. The physiological mechanism of the impact of the conditions of preincubation on maximal NPQ will be a subject of our future research.
Conclusions

The results obtained suggest that the increased content of $\alpha$-Toc and PQ$_{tot}$, as well as the increase in NPQ efficiency, play a role in the acquisition of copper tolerance. The role of light in the enhancement of copper-toxicity and the role of POX in response to elevated copper has also been shown.

Abbreviations

Asc, ascorbate; Car, carotenoids; Chl, chlorophyll; df, degrees of freedom; F, calculated statistics; $F_v/F_m$, maximum quantum yield of photosystem II; GSH, glutathione; NPQ, nonphotochemical quenching of chlorophyll fluorescence; p, probability of obtaining a result by chance; POX, peroxidase; PQ, plastoquinone; PQH$_2$, plastoquinol; PQ$_{tot}$, sum of plastoquinol and plastoquinone; PS II, photosystem II; ROS, reactive oxygen species; Tocs, tocopherols; $\alpha$-Toc, $\alpha$-tocopherol; $\gamma$-Toc, $\gamma$-tocopherol.

Declarations

Ethical Approval:

Not applicable.

Consent to Participate:

The authors approved their participation and the contribution described in the present manuscript.

Consent to Publish:

The authors approved the publication.

Availability of data and materials:

The data sets and the R scripts used are stored in the Open Science Framework repository (https://osf.io/gyq42/?view_only=6f556d7526394f7f808fa69be97d251f).

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Competing of interest:

The authors declare no conflicts of interest that are relevant to the content of this article.

Authors’ contributions:
References


Figures
Figure 1

Photosynthetic pigment content and peroxidase (POX) activity in the two experimental lines (N1 and Cu2) in relation to copper exposure in light and shade conditions: a) chlorophyll $a$, b) chlorophyll $b$, c) carotenoids concentrations, and d) peroxidase activity. The median values are presented with quartiles.

Figure 2
Prenyllipid content in the four experimental lines (Wall, N1, Cu2 and Cu200) in relation to a range of copper concentrations. **a)** a-Toc, **b)** total PQ (PQtot) **c)** PQH2, **d)** PQ, and **e)** PQ/PQtot. The median values are presented with quartiles. The prenyllipid content was normalized to the total Chl content and expressed in [mol/100 mol Chl a+ b].

**Figure 3**
Nonphotochemical chlorophyll fluorescence quenching (NPQ) over time in experimental lines (N1 and Cu2) in a) absence and b) presence of additional nutrients. Curves were plotted for all combinations of light or dark pretreatments and absence or presence of copper exposure.