Copper excess-induced large reversible and small irreversible adaptations in a population of *Chlamydomonas reinhardtii* CW15 (Chlorophyta)

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Abstract

Two *Chlamydomonas reinhardtii* CW15 populations modified by an excess of copper in growth medium were obtained: a “Cu” population that was continuously grown under the selection pressure of 5 µM Cu²⁺ (for at least 48 weeks) and the “Re” population, where a relatively short (9 week) exposure to elevated copper, necessary for acquiring tolerance, was followed by a prolonged period (at least 39 weeks) of cultivation at a normal (0.25 µM) copper concentration.

Cells of the Cu population were able to multiply at a Cu²⁺ concentration 16 times higher than that of the control population at a normal light intensity and at a Cu²⁺ concentration 64 times higher when cultivated in dim light. The potential quantum yield of photosystem II (Fv/Fm ratio) under copper stress was also significantly higher for the Cu population than for Re and control populations.

The Re population showed only residual tolerance towards the elevated concentration of copper, which is revealed by an Fv/Fm ratio slightly higher than in the control population under Cu²⁺ stress in dim light or in darkness.

We postulate that in the *Chlamydomonas populations* studied in this paper, at least two mechanisms of copper tolerance operate. The first mechanism is maintained during cultivation at a standard copper concentration and seems to be connected with photosynthetic apparatus. This mechanism, however, has only low adaptive value under excess of copper. The other mechanism, with a much higher adaptive value, is probably connected with Cu²⁺ homeostasis at the cellular level, but is lost during cultivation at a normal copper concentration.

Keywords

adaptation; *Chlamydomonas reinhardtii*; chlorophyll fluorescence; copper; heavy metals; microevolution

Introduction

Heavy metal pollution is an important global problem due to anthropogenic sources of pollution such as metallurgy and electroplating, chemical industries, dyes and pigments, ink manufacturing, paper mills, leather treatment, pharmaceuticals, textiles, and fertilizers [1].

Copper is an essential bioelement, however in excessive amounts it can be toxic. Deficiency symptoms in plants were observed when the copper content in vegetative...
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Plants protect themselves against copper excess in three ways: the extracellular complexation of metal excess (mainly by cell wall components); precipitation in the cytoplasm or vacuole; or binding by polypeptides or proteins [18]. It is well known [19] that protective mechanisms can be constitutive or inducible and that there are genetic differences between Cu-tolerant and Cu-sensitive organisms of the same species.

*Chlamydomonas reinhardtii* P. A. Dang is a model green alga for photosynthesis research and environmental stress investigations [20]. After complete sequencing of its genome in 2007, this organism became even more useful [21]. Many researchers have demonstrated heavy metal excess effects on *Chlamydomonas* growth, photosynthetic activity, and pigment content [22,23]. It has been shown that mutants of *C. reinhardtii* that lack a cell wall are more sensitive to Cd\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), and Ni\(^{2+}\) excess than the wild type [16,17,24].

Until now, heavy metal resistant populations of *C. reinhardtii* have been isolated and characterized physiologically for ballast elements only. Fujiwara et al. [25] raised a population that tolerates an arsene concentration at least 5 times higher than the parent strain, while two other research groups [26–28] obtained and characterized Cd\(^{2+}\) resistant populations. However, in available literature there are no data describing microevolutionary processes leading to formation of tolerance to copper ions. Unlike cadmium, in case of this metal the defense mechanism cannot be restricted to exclusion processes because copper, although very toxic at higher concentrations, is a necessary microelement and its uptake in certain amounts is of vital importance for cell functioning.

The aim of this study was to investigate the adaptive response of a population of *Chlamydomonas reinhardtii* to high copper concentrations. We used the cell wall deficient mutant CW15 as it was described by Nagel and Voigt [28] and Collard and Matagne [26,27], due to its higher sensitivity to heavy metal stress and the ease of cell fractionation. After obtaining *Chlamydomonas* cultures with increased Cu resistance, we characterized the basic differences in copper sensitivity and photosynthetic activity between Cu-sensitive and Cu-tolerant populations. The sensitivity of Cu-tolerant and paternal populations against the excess of both cadmium and zinc ions were also studied to check the specificity of generated resistance mechanisms.

**Material and methods**

**Experimental populations**

The cell wall deficient mutant (CW15) [29] of *Chlamydomonas reinhardtii* was obtained from Dr. Itzhak Ohad, Hebrew University (Department of Biological Chemistry, Givat Ram, Jerusalem, Israel) in the 1990s. It was aseptically cultured in Erlenmeyer flasks (250 mL) on a Sager–Granick medium [30], supplemented with 100 mM mannitol as an osmoprotectant and sodium acetate (75 mM) and citrate (1.7 mM) as sources of...
organic carbon. Cultures were gently shaken in a growth chamber at 22 ±2°C under continuous light (50 μmol photons m⁻² s⁻¹ photosynthetically active radiation – PAR) from fluorescent lamps.

The Cu²⁺-adapted population (“Cu”) was cultivated on Sager–Granick medium with Cu²⁺ concentration elevated to 5.25 μM for 57 weeks to generate copper tolerance. The Cu²⁺-modified revertant population (“Re”) was adapted to elevated copper concentration for 9 weeks and then was cultivated for 48 weeks on a medium containing a nominal concentration (0.25 μM) of Cu²⁺ as a micronutrient. The control (“K”) population was cultivated at a Cu²⁺ concentration of 0.25 μM, nominal for Sager–Granick medium, and was never stressed by copper excess. During experiments, 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).

Tolerance to metals

The microcultures used for testing tolerance of increasing concentrations of copper (0.5–250 μM, as CuSO₄), cadmium (0–128 μM, as CdSO₄), or zinc (3.8–260 μM, as ZnSO₄) ions were grown aseptically under a light intensity of 28 μmol m⁻² s⁻¹ of PAR in standard 96-well flat-bottom plates, without shaking. An additional experiment tested the effect of three light regimes: “normal” – 28 μE m⁻² s⁻¹; “dim” – 6.25 μE m⁻² s⁻¹ continuous white light (fluorescent lamps); and “dark” – continuous darkness, on the growth of studied populations in a gradient of Cu²⁺ concentration. In all cases, at least three independent replicates for each metal concentration were obtained.

Measurements of algae growth and photosynthetic activity

Photosynthetic pigment concentrations in algal suspensions were measured in an 80% acetone extract using the Lichtenthaler [31] method with a Jasco 870 UV-Vis spectrophotometer (Jasco, USA) at wavelengths of 470, 646.6, 662.2, and 710 nm in a glass cuvette with a 1-cm optical path.

Fluorescence parameters were measured using a PAM-101 chlorophyll fluorometer (Walz, Germany) with a red (600–680 nm) saturating light source (5,000 μmol photon m⁻² s⁻¹). The output of the light guide was focused on the bottom of the plate well using a microscope condenser (PZO, Poland) to obtain maximum fluorescence signal intensity and a light spot of such diameter as to fit the internal diameter of the well bottom.

For a quick estimation of chlorophyll content in the well, the maximal chlorophyll fluorescence intensity (Fₘ) measured by PAM 101 (average of three measurements) was used. The relationship between Fₘ and chlorophyll concentration in the culture, measured spectrophotometrically, was rectilinear for low concentrations (up to 20 mg L⁻¹). Higher pigment concentrations and a clustered culture growth pattern made this interdependence curvilinear, but Fₘ still increased with increasing pigment concentrations (Fig. 1). We decided to use this method to semi-quantitatively estimate the algae growth rate because the method is fast, reliable, and noninvasive. Fluorescence parameters were measured in 5–8-day-old cultures for time-dependent experiments and on the third day of culture for light-intensity experiments, when the Fₘ and F₀ (chlorophyll fluorescence in the absence of an actinic light) signals allowed the full utilization of the PAM-101 detector dynamic range.

Statistical analyses

Algal microculture growth and photosynthetic activity were analyzed using general linear mixed model [32]. In all cases, the experimental population was a fixed factor, while log-transformed metal concentration and time were continuous predictors.
When the effects of light were analyzed, it was entered as a fixed factor. In all analyses, the replicate culture was entered as a random effect (results not shown). Initially, each statistical model included all interactions of the main effects. The nonsignificant interactions were sequentially removed from the models to increase power of the tests. In case of significant higher-order interactions, we performed the analyses of differences in experimental populations separately for each metal concentration (see supplementary material) or light condition. If relevant, we performed post hoc analyses to reveal significant differences between the experimental populations. To normalize distribution of the data, we used the following transformations of the dependent variable, for algae growth: log $F_M$ and for photosynthetic activity $\log(1 - F_V/F_M)$. Graphs present data without transformation. Analyses were performed in SAS Enterprise v.6.1.

Results

Experimental populations

In the first stage of the study, we determined the maximal concentration of copper ions in the medium that was not lethal for *Chlamydomonas reinhardtii* CW15 ("CW15") cultures. We found that the highest concentration of Cu$^{2+}$ in the medium that permits the completion of the life cycle of nonadapted algae was 5.25 µM, although this did cause a strong decrease in the rate of cell multiplication (data not shown). After nine passages (= 9 weeks) of culture using a medium containing 5.25 µM Cu$^{2+}$, we found that the multiplication rate (measured as $F_M$ intensity change) of the Cu-adapted population was much higher than that of nonadapted algae in the medium containing the same concentration of copper. At this stage, the Cu-adapted population was divided into two parts. The first one was continuously cultivated on a Cu$^{2+}$-rich (5.25 µM Cu$^{2+}$) medium (the Cu population), while the other was cultured on a standard Sager–Granick medium (0.25 µM Cu$^{2+}$; the revertant Re population) to check the stability of the adaptation. The results for Re population presented below were obtained using algae samples harvested from this population after at least 48 passages (= 48 weeks) of further cultivation on standard medium. Samples of a control (K) population, which was never been stressed by copper excess, were also examined.

Tolerance to copper

The sensitivity of the Cu, Re, and K populations to copper in terms of growth dynamics (measured as $F_M$) and photosynthetic activity (measured as $F_V/F_M$) was analyzed in 5–8-day-old cultures, which were grown in varying Cu$^{2+}$ concentrations under standard light intensity.

We found that variation in algae growth was shaped by the interactions of experimental population and Cu$^{2+}$ concentration as well as experimental population and time (Tab. 1). Analyses performed separately for each Cu$^{2+}$ concentration revealed that, with increasing metal concentration, the growth of the three experimental populations became more diverse (Tab. S1, Fig. 2). Specifically, there were no differences in the growth of experimental populations in control conditions (0.25 µM Cu$^{2+}$); in Cu$^{2+}$ concentrations of 0.5–0.75 µM, the experimental populations differed but their growth rate was similar (i.e., parallel in time), while at higher Cu$^{2+}$ concentrations, significant differences between experimental populations in their growth rate as a function of time were found (as indicated by significant interaction term). The growth rate of the Cu population remained the same as control up to a concentration of 8.25 µM of Cu$^{2+}$. For the K and Re populations, there was significant decrease of the $F_M$ signal in comparison with Cu population at copper ion concentrations higher than 0.75 µM. At high copper ion concentrations (higher than 4.25 µM for K and Re and 64 µM for Cu populations) a very weak or negligible $F_M$ signal was detectable. In general, the sensitivity of the growth rate of both K and Re populations to copper excess was similar and it was far higher than that of the Cu population (Fig. 2). The effect of copper ions on algae photosynthetic activity ($F_V/F_M$) of these populations under the same experimental conditions
Tab. 1 Analyses of algae growth (log $F_M$) and photosynthetic activity [$\log(1 - F/V/F_M)$] in relation to experimental population, log Cu concentration, and time.

| Factor                                         | $F_M$  | $p$     | $1 - F/V/F_M$ | $p$     |
|------------------------------------------------|--------|---------|---------------|---------|
| Experimental population                        | 2.74   | 0.0661  | 8.03          | 0.0004  |
| Log Cu concentration                           | 24.45  | <0.0001 | 61.77         | <0.0001 |
| Time                                           | 113.32 | <0.0001 | 20.26         | <0.0001 |
| Experimental Population × Log Cu Concentration | 490.49 | <0.0001 | 18.03         | <0.0001 |
| Experimental Population × Time                 | 4.90   | 0.0081  | 10.51         | <0.0001 |
| Log Cu Concentration × Time                    | 4.89   | 0.0279  | 27.20         | <0.0001 |
| Experimental Population × Log Cu Concentration × Time | 16.00 | <0.0001 |

Fig. 2 Growth of *Chlamydomonas reinhardtii* CW15 cultures on media with different Cu$^{2+}$ concentration, measured as $F_M$. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population.
was more complicated and involved three-way interaction (Tab. 1). At the lowest Cu\(^{2+}\) concentrations (0.25–0.5 µM), the photosynthetic activity of all algae populations was the same on each of the measurement days (Tab. S2, Fig. 3). At 0.75 and 1.25 µM Cu\(^{2+}\), the three experimental populations differed slightly from each other, such that Re showed the highest photosynthetic activity, but they did not change significantly with time. Intermediate concentrations of copper ions (2.25 and 4.25 µM) led to a significantly different algae photosynthetic activity of the experimental populations in subsequent days, such that K population showed the lowest starting level followed by a dynamic increase, especially in 4.25 µM Cu\(^{2+}\). At 8.25 µM Cu\(^{2+}\), K population showed a significant decline in the F\(_{V}/F_{M}\) parameter, whereas in Re population, this was not measurable for the duration of the experiment because of the low F\(_{O}\) (ground fluorescence) signal. On the other hand, F\(_{V}/F_{M}\) for Cu population remained relatively stable in time and only slightly decreased with increasing copper concentrations.

**Effects of light conditions**

The effects of Cu\(^{2+}\) concentrations on the three investigated CW15 populations were characterized under three different light regimes. We measured the algae growth (Fig. 4) and F\(_{V}/F_{M}\) (Fig. 5) of cultures grown under normal light (28 µmol m\(^{-2}\) s\(^{-1}\) of PAR), dim light (6.25 µmol m\(^{-2}\) s\(^{-1}\) of PAR), and in darkness.

The most distinguished feature of the algal cultures grown under the three light regimes was that there was a synergistic effect of light and an excess of Cu\(^{2+}\) indicated by the significant interaction term (Tab. 2). Under normal light conditions, the three experimental populations showed similar growth in the range of low Cu\(^{2+}\) concentrations (0.25–1.25 µM). Higher concentrations caused a significant decline of growth in
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**Fig. 4** *Chlamydomonas reinhardtii* CW15 culture densities (measured as $F_M$, arbitrary units) after 3 days of growth on media with different Cu$^{2+}$ content and under three light regimes: normal – 28 µmol m$^{-2}$ s$^{-1}$ of PAR, continuous light; dim – 6.25 µmol m$^{-2}$ s$^{-1}$ of PAR, continuous light; dark – continuous darkness. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population. Pay attention to the FM scales!

**Fig. 5** Maximal quantum efficiency of photosystem II ($F_V/F_M$) in *Chlamydomonas reinhardtii* CW15 cultures in third day after inoculation on media with different Cu$^{2+}$ concentration and under three light regimes: normal – 28 µmol m$^{-2}$ s$^{-1}$ of PAR, continuous light; dim – 6.25 µmol m$^{-2}$ s$^{-1}$ of PAR, continuous light; dark – continuous darkness. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population.

**Tab. 2** Analyses of algae growth (log $F_M$) and algae photosynthetic activity [log($1 - F_V/F_M$)] in relation to light conditions, experimental population, and log Cu concentration.

|                                | log $F_M$ | p     | log($1 - F_V/F_M$) | p     |
|--------------------------------|-----------|-------|--------------------|-------|
| **Light conditions**           |           |       |                    |       |
| 108.38$^{a}_{304}$             | <0.0001   |       | 502.89$^{a}_{245}$  | <0.0001|
| **Experimental population**    |           |       |                    |       |
| 0.23$^{a}_{304}$               | 0.7961    |       | 4.82$^{a}_{245}$    | 0.0108 |
| **Log Cu concentration**       |           |       |                    |       |
| 775.43$^{a}_{304}$             | <0.0001   |       | 140.26$^{a}_{245}$  | <0.0001|
| **Log Cu Concentration × Experimental Population** |           |       |                    |       |
| 4.60$^{a}_{304}$               | 0.0107    |       | 20.26$^{a}_{245}$   | <0.0001|
| **Experimental Population × Light Conditions** |           |       |                    |       |
| 11.35$^{a}_{304}$             | <0.0001   |       | 17.07$^{a}_{245}$  | <0.0001|
| **Log Cu Concentration × Light Conditions** |           |       |                    |       |
| 62.59$^{a}_{304}$             | <0.0001   |       | 56.23$^{a}_{245}$   | <0.0001|
| **Experimental Population × Light Conditions × Log Cu Concentration** |           |       |                    |       |
| 12.28$^{a}_{245}$             | <0.0001   |       |                    |       |
the K and Re populations, whereas the Cu population was more resistant to copper excess: we observed the decline in growth at concentrations higher than 4.25 µM Cu²⁺ (Fig. 4). In dim light, the sensitivity of all experimental populations against copper excess was lower than in normal light. This was supported by the significant interaction of experimental populations and Cu²⁺ concentrations (Tab. 3). Under dim light, the K and Re populations showed a progressive decrease in population density from the maximum value to zero, along with an increase in copper concentration. In darkness, the growth of all populations was significantly reduced (Fig. 4) and it declined with increasing Cu²⁺ concentration. The highest and most stable growth over the wide range of Cu²⁺ concentrations (0.25–64 µM Cu²⁺) was observed in the Cu population. The Cu population under all light regimes were more tolerant to high copper concentrations than Re and K populations, although at Cu²⁺ concentrations lower than 1.25 µM, the Cu culture densities were below those of the K and Re populations.

The photosynthetic activity (Fₐ/Fₚₛ) in normal light was significantly lower for K than for both adapted populations, even in the control medium (Fig. 5). In copper excess, the inhibition of photosynthetic activity was also stronger for the K population, whereas the Cu population seemed to be much more tolerant. Interestingly, within the high range of concentrations of Cu²⁺ (>8.25 µM), the photosynthetic activity of all three populations was higher and similar. In dim light, there was only a weak and uniform decay of Fₐ/Fₚₛ in all populations within a concentration range of 0.25–4.25 µM, whereas at higher concentrations in the Re and K populations, there was strong inhibition and the effect was larger for the K population. The Cu population, by contrast, maintained activity similar to control levels up to 16.25 µM Cu²⁺ and, at higher concentrations, there was only a mild inhibition of photosynthetic activity. In total darkness, both Cu and Re populations displayed similar symptoms as in dim light, except that in Cu cultures, even at the highest copper concentration, there was no significant inhibition of Fₐ/Fₚₛ. In contrast, in the K population, there was strong and progressive inhibition within the whole range of Cu²⁺ concentrations used. High variation of the Fₐ/Fₚₛ parameter in darkness (Fig. 5) is the likely reason why the interaction between the experimental population and Cu²⁺ concentration was not significant in darkness, while it was in the two other light conditions (Tab. 3).

**Tolerance to cadmium and zinc**

Finally, we compared the population growth response and Fₐ/Fₚₛ in copper-adapted and control population of CW15 to cadmium and zinc ions excess.
Variation in algae growth measured in relation to Cd\textsuperscript{2+} concentration (Fig. 6 cf. Fig. 2; Tab. 4) revealed that there was no interaction between the experimental population and metal concentration. At concentrations between 32–64 µM Cd\textsuperscript{2+}, there was a 50% decrease in all population densities on the sixth day of culture, as was approximated from the whole data set (not shown). It can be interpreted that there are nonsignificant differences in general sensitivity between the algae populations used in Cu\textsuperscript{2+} adaptation experiments against various Cd\textsuperscript{2+} concentrations. On the other hand, experimental population type and metal concentration showed significant interaction with time. This means there are statistically significant differences in growth dynamics between experimental populations as well as within the same algae population in different cadmium concentrations. Pair-wise comparisons between experimental populations (controlling for the effects presented in Tab. 4) revealed that the Re population had significantly slower growth compared to Cu (p = 0.0010) and K (p = 0.0124) populations, while Cu population did not differ from population K (p = 0.3826; see also Fig. 6).

Variation in F\textsubscript{v}/F\textsubscript{m} was shaped by three two-way interactions of the main factors (Tab. 4). Analyses performed separately for each Cd\textsuperscript{2+} concentration showed that the strongest effects of experimental population and time were observed in the highest concentration of cadmium (Tab. S3, Fig. 7).

Variation in algae growth and algae photosynthetic activity measured in relation to Zn\textsuperscript{2+} concentration revealed that both parameters are shaped by significant interactions between the experimental populations and metal concentration (Tab. 5). Pair-wise comparisons of growth between all experimental populations at two zinc concentrations revealed that only the growth of Cu population differed significantly between concentrations (p < 0.0001), meaning it was inhibited in comparison to K and Re populations in the higher Zn\textsuperscript{2+} concentration (Fig. 8). The other populations showed
Tab. 4  Analyses of algae growth (log $F_M$) and algae photosynthetic activity [log($1 - F_V/F_M$)] in relation to experimental population, log Cd concentration, and time.

|                         | log $F_M$ | log($1 - F_V/F_M$) |
|-------------------------|-----------|---------------------|
|                         | $F_{d}$   | $p$                 | $F_{d}$   | $p$                 |
| Experimental population | 3.72, 336 | 0.0253              | 6.04, 336 | 0.0026              |
| Log Cd concentration    | 24.71, 336| <0.0001             | 48.66, 336| <0.0001             |
| Time                    | 39.31, 336| <0.0001             | 0.03, 336 | 0.8683              |
| Log Cd Concentration x Time | 6.62, 336 | 0.0105              | 18.44, 336| <0.0001             |
| Experimental Population x Time | 4.44, 336 | 0.0125              | 5.36, 334 | 0.0051              |
| Experimental Population x Log Cd Concentration | 73.03, 334 | <0.0001             |

Fig. 7  Changes of maximal quantum efficiency of photosystem II ($F_V/F_M$) from fifth to eight day after inoculation in *Chlamydomonas reinhardtii* CW15 cultures on media with different Cd$^{2+}$ concentration. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population. The control without Cd$^{2+}$ – see Fig. 3, 0.25 µM Cu$^{2+}$.
no significant differences. Pair-wise comparisons of $F_{V}/F_{M}$ between all experimental populations at two Zn$^{2+}$ concentrations revealed that all populations showed lower potential photosynthetic activity at higher concentrations (all $p < 0.0116$; Fig. 9).

**Tab. 5** Analyses of algae growth (log $F_{M}$) and algae photosynthetic activity [log($1 - F_{V}/F_{M}$)] in relation to experimental population, Zn concentration, and time.

|                         | log $F_{M}$ |   | log($1 - F_{V}/F_{M}$) |   |
|-------------------------|-------------|---|------------------------|---|
|                         | $F_{df}$    | $p$ | $F_{df}$    | $p$ |
| Experimental population | 1.89, 0.65  | 0.1588 | 0.57, 0.75  | 0.5673 |
| Zn concentration        | 7.68, 0.65  | 0.0073 | 16.06, 1.72 | 0.0001 |
| Time                    | 34.97, 0.65 | <0.0001 | 2.62, 1.72  | 0.1102 |
| Experimental Population × Zn Concentration | 5.34, 0.65  | 0.0071 | 6.19, 1.72  | 0.0033 |
| Zn Concentration × Time | 4.68, 0.65  | 0.0342 | 6.19, 1.72  | 0.0152 |

**Fig. 8** Growth of *Chlamydomonas reinhardtii* CW15 cultures on media with elevated Zn$^{2+}$ concentration, measured as $F_{M}$. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population. The control with standard Zn$^{2+}$ concentration – see Fig. 2, 0.25 µM Cu$^{2+}$.

**Fig. 9** Changes of maximal quantum efficiency of photosystem II ($F_{V}/F_{M}$) from fifth to eighth day after inoculation in *Chlamydomonas reinhardtii* CW15 cultures on media with elevated Zn$^{2+}$ concentration. K – control population; Cu – Cu$^{2+}$-adapted population; Re – revertant population. The control with standard Zn$^{2+}$ concentration – see Fig. 2, 0.25 µM Cu$^{2+}$. 
Discussion

The primary goal of this work was to study two populations of *Chlamydomonas reinhardtii* CW15 modified by excess copper in the growth medium: Cu population that was continuously grown under the selection pressure of 5 µM Cu²⁺ and Re population that was grown for a relatively short (9 weeks) period in copper excess, necessary for the build-up of tolerance, followed by a prolonged period of cultivation at a normal copper concentration.

Methods of experimental evolution allow the generation of organisms with specific physiological properties in order to investigate mechanisms of adaptation [33,34]. To date, algae have been the subject of a number of such studies. Pre-exposure of *Euglena gracilis* for 55–60 generations to mercury or cadmium ions induced enhanced tolerance for excess of metal ions [35]. Nagel and Voigt [28] obtained *Chlamydomonas reinhardtii* CW15, which is resistant to cadmium ions, by culturing this alga in continually increasing Cd²⁺ concentrations. Tolerant cells were able to multiply even at 300 µM Cd²⁺ (wild cells at 70 µM). To our best knowledge there was no investigation of algae experimental evolution on micronutrient elements, including copper. On the other hand, adaptation to high Cu²⁺ concentrations was observed in natural conditions in several algal species [36–38]. Adaptation of biological populations to environmental changes in multigenerational time scales can be achieved by structural and functional changes at different levels of the system [39–41]: (i) adaptation of specimens within the framework of phenotypic plasticity; (ii) epigenetic changes; (iii) rearrangement of alleles of different genes responsible for resistance to heavy metal stress to form phenotypes with better fitness under new conditions (recombinative variability); and (iv) selection of existing populations, or newly formed alleles of sparse genes necessary for adaptation to the new environment.

Adaptation at the specimen level is reversible by nature, but in our investigation, we did not observe these reversible effects, due to the time scale of this phenomenon. The reversion is also observed when a population is adapted to environmental stress conditions through the mechanism of recombined genes which return to the initial state in absence of stressor. A similar result will be also obtained when adaptive changes are at the epigenetic level. On the other hand, the mechanism of allele selection would irreversibly change the genetic structure of a population, in which most specimens are haploidal, as in *Chlamydomonas*, if the specific allele of particular gene is necessary to survive.

In the Cu population, all possible mechanisms of copper excess tolerance were induced and kept active. In the Re population, adaptive changes were conserved only if they were necessary to survive in the face of excess Cu²⁺. In this case, other alleles of respective genes would be eliminated and/or changes in allele frequency would be conserved because of their neutral character in the absence of copper excess [42].

Copper excess is a well-known factor that causes a decrease in growth rate and chlorophyll concentration in cultures of *Chlamydomonas reinhardtii* [17,24,43] and other micro- and macroalgae [44]. Luis et al. [22] demonstrated that high Cu²⁺ concentrations (>50 µM) are able to disturb cell division in *Chlamydomonas* by the inhibition of the transcription of cyclin-dependent protein kinase (CDK), which is necessary for this process. Other effects of copper excess are also described in this paper. Devriese et al. [45] reported the inhibition of nitrate assimilation under excess copper concentrations.

Plants and algae can regulate metal ion concentrations and prevent undesirable excess effects. Concentrations are regulated by adjusted uptake, cytosolic concentrations, and redistribution between vacuole and different organelles [46]. The same phenomena were described in *Chlamydomonas reinhardtii* as mechanisms of copper homeostasis [20,47] and as a response to heavy metal excess (for review see [48]). Nagel and Voigt [28] showed that, in *Chlamydomonas reinhardtii* CW15 resistant to cadmium ions, cells of the tolerant population bound more metal ions than those of the sensitive population. The results of other selection experiments [26] aimed at obtaining tolerance to Cd²⁺ showed that, in original *C. reinhardtii* populations, there were subpopulations with a gene responsible for low tolerance. In a strain selected on media containing higher concentrations of Cd²⁺, the authors observed mutation connected with a reduction in sensitivity to cadmium. Both mutations (one already present in populations and a new one) were dominant. Mechanisms of resistance to excess copper stress in the green macroalga *E.*
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Compressa operated at both the hereditary and phenotypic level [37]. Because heavy metals alter the redox balance, one of the most important protective mechanisms is maintaining the high antioxidative potential of cells and their compartments [49].

Our results demonstrate that the Cu population exhibits much higher tolerance than the K population to an excess of copper. The adaptation is so strong that the cells of this population are able to complete their life cycle in a medium containing a copper concentration 16 times higher than control or Re were able to complete their life cycles (Fig. 2, Fig. 4). Although the multiplication of cells at a high Cu²⁺ concentration (8.25 µM) was significantly slower than at a normal concentration of these ions, the culture density that could be attained at the plateau stage would be at least as high as in control medium (Fig. 2). On the other hand, concentrations of copper up to 8.25 µM in culture media have no significant influence on the potential photosynthesis quantum yield (F₅/Fₐ) of the Cu population, whereas it was strongly inhibited at that concentration in the control population (Fig. 3). The F₅/Fₐ in the Re population showed only slightly lower resistance than Cu at 4.25 µM of Cu²⁺, whereas population growth was strongly inhibited (Fig. 2, Fig. 3).

Photosynthetic activity is one of the most sensitive sites of the photoautotrophic metabolism (for review see [50]) and photosystem II is seen as the main target for copper action, mainly at the donor side [51], but also on the acceptor side [44] or at the cytochrome b₅₅₃ site [52]. Liu et al. [53] have additionally shown that Cu-substituted chlorophyll can be incorporated into the PSII structure and disturb the excitation migration processes in this complex.

The pro-oxidative action of copper ions in algal photosynthetic systems has been reported by many researchers [54–56]. Lupi et al. [43] described increased copper ion toxicity (40 µM Cu²⁺) when light intensity was increased from 100 to 150 W m⁻² in Chlorella vulgaris cultures. The decrease of Cu²⁺ toxicity under low light may be the result of decreased electron transport rate and dioxygen formation in thylakoid membranes. It is of interest that in Chlamydomonas, the copper level and oxygen level signaling are linked [5]. It seems that in C. reinhardtii CW15 the cell division processes are highly sensitive to excess copper under continuous light – the same populations are able to multiply in copper concentrations several times higher when cultured in a 14:10 h light:dark cycle (Pluciński et al., 2018, manuscript in preparation).

The multiplication rate of the Re population growing in normal light conditions was not significantly different from the control (Fig. 4). On the other hand, residual adaptation was visible as the higher resistance of the photosynthetic apparatus (Fig. 5), which was also manifested under mild copper stress (0.75–4.25 µM) between the fifth to eight days of culture under normal light (Fig. 3). As in the case of Cu population, the resistance of photosynthetic apparatus in 3-day-old Re cultures in darkness at lower copper concentrations (Fig. 5) is similarly strong. However, there is no pronounced resistance at high Cu²⁺ concentrations under weak light, and within a low to medium range of copper excess under normal light.

A mechanism that is active in the photosynthetic apparatus of the Re population in darkness and protects this system against the ROS that can be produced by respiratory (reverse) electron transport in the photosynthetic membranes of Chlamydomonas should be considered [57]. This is quite possible because in heterotrophically grown plant cells mitochondrial electron transport can be an important source of ROS [58].

It seems that the mechanism responsible for excess copper tolerance in terms of population growth is very specific to Cu²⁺ because the adapted Cu population does not show any increased resistance to Cd²⁺ or Zn²⁺ (Fig. 6, Fig. 8).

From a biochemical and physiological point of view, copper, cadmium, and zinc belong to different groups of heavy metals. In low concentrations, zinc is an important micronutrient (while cadmium is a ballast element). Accordingly, we observed an increasing growth rate in algae cultivated in low concentrations of Zn²⁺, as reported by Lin and Aarts [59].

Cu²⁺ can generate ROS in a direct process via the Fenton reaction [60]. In contrast to copper, cadmium ions generate ROS species mainly by indirect reactions: by a reaction with electron transport chain proteins [61] or by a decrease of antioxidative enzymatic activity [62]. In our cultures, we did not observe any relationship between copper tolerance and the ability to maintain a high growth rate and efficiency of photosynthesis at high concentrations of Cd²⁺ and Zn²⁺. Some investigators have observed element-specific
formation of particular mechanisms of defense: phytochelatin synthesis was induced by Cd\(^{2+}\) but not by Cu\(^{2+}\) in Micrasterias denticulata [63] and only cadmium was able to induce an increase of acid-soluble thiol levels in Euglena gracilis adapted to mercury or cadmium [35].

On the other hand, the activity of the photosynthetic apparatus of the Cu population seems to be slightly more sensitive to a high concentration of cadmium ions, whereas in the Re population, some resistance mechanism is present. This led to no change in F\(_{V}/F\(_{M}\) ratio in low concentration of Zn\(^{2+}\) and in lower concentrations of Cd\(^{2+}\) when compared with the control medium (Fig. 3, Fig. 7, and Fig. 9).

The present data show that, in resistance to excess copper in CW15, at least two mechanisms are involved. The first protects all metabolic processes in the whole cell. Excess copper induces this complex mechanism, which reverts under cultivation at a normal copper concentration. This mechanism likely removes excess Cu\(^{2+}\) from the cell and possibly induces its deactivation within the cell. However, the process is likely metabolically expensive and/or disturbs copper homeostasis in normal conditions. For these reasons, the mechanism would be turned off if it is only physiologically regulated, or it would be microevolutionarily eliminated if it has epigenetic and/or recombinant genetic background, when it is no longer necessary.

The second mechanism is probably connected with the structure of the photosynthetic apparatus and it is genetically fixed. It seems that, at optimal Cu\(^{2+}\) concentrations, the fitness of the cells that use this mechanism is not significantly lower than that of the control population cells. We propose that this mechanism may involve changes in the structure of the photosynthetic apparatus.

Conclusions

The population of Chlamydomonas reinhardtii CW15, tolerant to excess copper ion, was obtained as the effect of a long period of culture under an elevated (5 µM) copper concentration.

We postulate at least two mechanisms of tolerance: one of them is maintained during cultivation in standard copper concentrations and seems to be connected with photosynthetic apparatus but has limited adaptive value. The other, with a much higher adaptive value, is likely connected with Cu\(^{2+}\) homeostasis at the cellular level but is lost during cultivation at a normal copper concentrations.

The investigated mechanism of Cu tolerance is metal-specific. The Chlamydomonas cells which acquired tolerance to copper are sensitive to zinc and cadmium toxicity.

These are the first data describing microevolutionary processes involved in Cu tolerance in Chlamydomonas reinhardtii CW15.

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Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3569/0:

Tab. S1 Analyses of algae growth (log F\(_{M}\)) in relation to experimental population and time analyzed for each Cu concentration separately.

Tab. S2 Analyses of algae photosynthetic activity [log(1 – F\(_{V}/F\(_{M}\)) in relation to experimental population and time analyzed for each Cu concentration separately.

Tab. S3 Analyses of algae photosynthetic activity [transformed by log(1 – F\(_{V}/F\(_{M}\)) in relation to experimental population and time analyzed for each Cd concentration separately.
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