View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Jagiellonian Univeristy Repository

Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3569

Publication history
Received: 2017-03-30
Accepted: 2018-01-03
Published: 2018-03-28

Handling editor
Paweł Sowiński, Faculty of Biology, University of Warsaw, Poland

Authors' contributions
BP contributed to all experimental and publishing process; AW was involved in design of the experiments and contributed to writing the manuscript; JR performed statistical analysis of the obtained results; KS contributed to preparation of the final version of the manuscript; all authors were engaged in discussion of the results

Funding
This work was financed from the budget of the Polish Ministry of Science for 2004–2008 under project No: 2P04C 020 27.

Competing interests
No competing interests have been declared.

Copyright notice
© The Author(s) 2018. This is an Open Access article distributed under the terms of the Creative Commons Attribution License, which permits redistribution, commercial and noncommercial, provided that the article is properly cited.

Citation
Pluciński B, Waloszek A, Rutkowska J, Strzałka K. Copper excess-induced large reversible and small irreversible adaptations in a population of *Chlamydomonas reinhardtii* CW15 (Chlorophyta). Acta Soc Bot Pol. 2018;87(1):3569. https://doi.org/10.5586/asbp.3569

Digital signature
This PDF has been certified using digital signature with a trusted timestamp to assure its origin and integrity. A verification trust dialog appears on the PDF document when it is opened in a compatible PDF reader. Certificate properties provide further details such as certification time and a signing reason in case any alterations made to the final content. If the certificate is missing or invalid it is recommended to verify the article on the journal website.

ORIGINAL RESEARCH PAPER

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

Bartosz Pluciński[1], Andrzej Waloszek[1], Joanna Rutkowska[2], Kazimierz Strzałka[1,3]*

[1] Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland
[2] Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland
[3] Małopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7A, 30-387 Cracow, Poland

* Corresponding author. Email: kazimierzstrzalka@gmail.com

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 Cu^{2+} (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 Cu^{2+} concentration 16 times higher than that of the control population at a normal light intensity and at a Cu^{2+} concentration 64 times higher when cultivated in dim light. The potential quantum yield of photosystem II (F_V/F_M 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 F_V/F_M ratio slightly higher than in the control population under Cu^{2+} 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 Cu^{2+} 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

tissues was lower than 5 mg kg^{-1} dry weight (DW), with toxic effects being observed above 20 mg kg^{-1} DW [2]. The metalloproteins containing copper ions – plastocyanin and cytochrome *c* oxidase – play an important role in energy transducing systems. They are also involved in the protection of cellular components against oxidation by reactive oxygen species (ROS). The best-known example is Cu and Zn ion-containing superoxide dismutase (CuZn-SOD) [3,4]. There are numerous reports on the mechanisms involved in the maintenance of copper homeostasis in plants [5–7]. However, excess of Cu^{2+} can negatively impact upon plant metabolic activity, including the damage of photosystems and enhanced ROS formation [8–10]. The high ability of Cu^{2+} ions to promote ROS generation is a result of catalytic action in Fenton-type reactions and/or the Haber–Weiss cycle. Toxic effects induced by elevated copper concentration can be enhanced by strong light [11,12]. It is well known that for plants [13–15] and green algae [16,17], a high level of bioavailable copper in the environment is more damaging than the presence of other heavy metal ions, such as Cd^{2+}, Pb^{2+}, or Zn^{2+}, which do not have redox capacity. This means they can only indirectly contribute to oxidative stress by reacting with proteins or by decreasing the concentration of low molecular weight antioxidants such as glutathione, ascorbate, or tocopherol.

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 arsenate 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, Givet 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 Cu^{2+}-adapted population ("Cu") was cultivated on Sager–Granick medium with Cu^{2+} concentration elevated to 5.25 μM for 57 weeks to generate copper tolerance. The Cu^{2+}-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 Cu^{2+} as a micronutrient. The control ("K") population was cultivated at a Cu^{2+} 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 $CuSO_4$), cadmium (0–128 μM, as $CdSO_4$), or zinc (3.8–260 μM, as $ZnSO_4$) 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 Cu^{2+} concentration. In all cases, at least three independent replicates for each metal concentration were obtained.

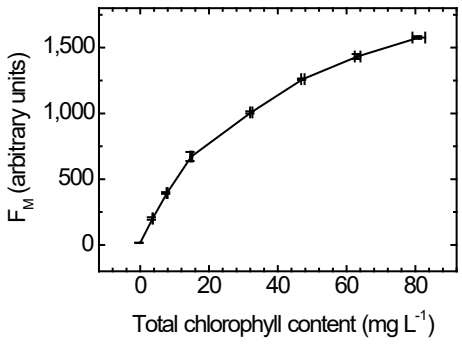

Fig. 1 Dependence between the maximal fluorescence (F_M, measured by PAM 101 chlorophyll fluorimeter; arbitrary units) of chlorophyll *a* in *Chlamydomonas reinhardtii* suspension in a single well of multiwell plate and total chlorophyll concentration extracted from the sample.

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 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 (F_M) measured by PAM-101 (average of three measurements) was used. The relationship between F_M 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 F_M 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 F_M and F_O (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.

	log F_M		log(1 – F_V/F_M)	
	F_{df}	p	F_{df}	p
Experimental population	$2.74_{2, 269}$	0.0661	$8.03_{2, 308}$	0.0004
Log Cu concentration	$24.45_{1, 269}$	<0.0001	$61.77_{1, 308}$	<0.0001
Time	$113.32_{1, 269}$	<0.0001	$20.26_{1, 308}$	<0.0001
Experimental Population × Log Cu Concentration	$490.49_{2, 269}$	<0.0001	$18.03_{2, 308}$	<0.0001
Experimental Population × Time	$4.90_{2, 269}$	0.0081	$10.51_{2, 308}$	<0.0001
Log Cu Concentration × Time	$4.89_{2, 269}$	0.0279	$27.20_{1, 308}$	<0.0001
Experimental Population × Log Cu Concentration × Time			$16.00_{2, 308}$	<0.0001

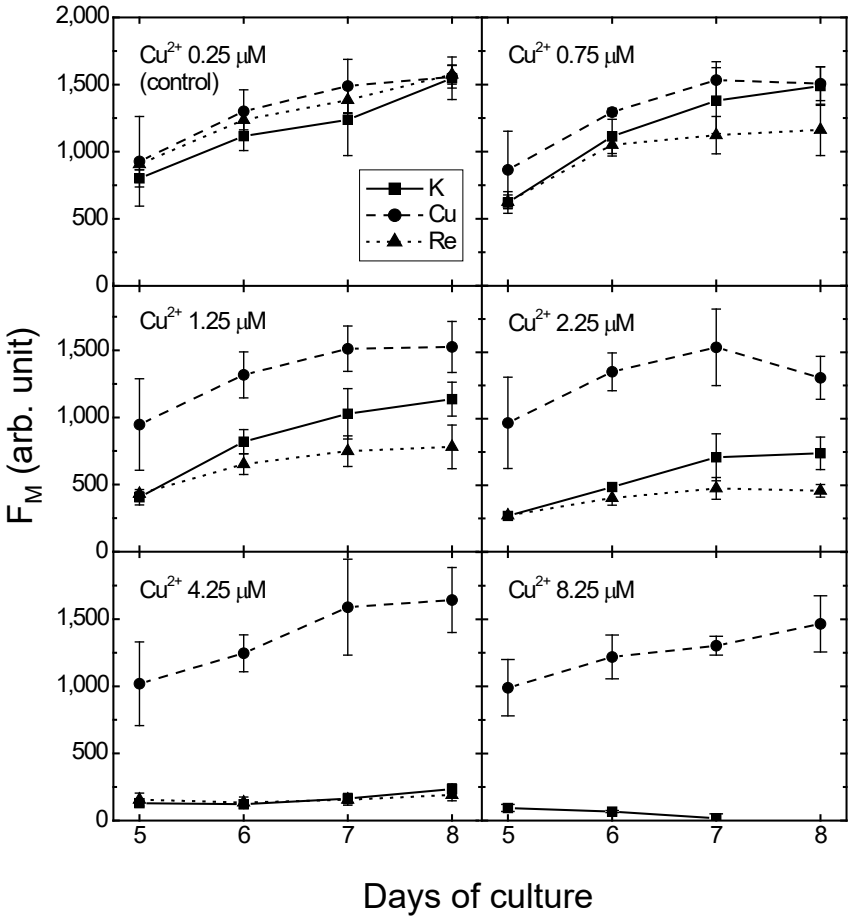

Fig. 2 Growth of *Chlamydomonas reinhardtii* CW15 cultures on media with different Cu^{2+} concentration, measured as F_M. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population.

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.

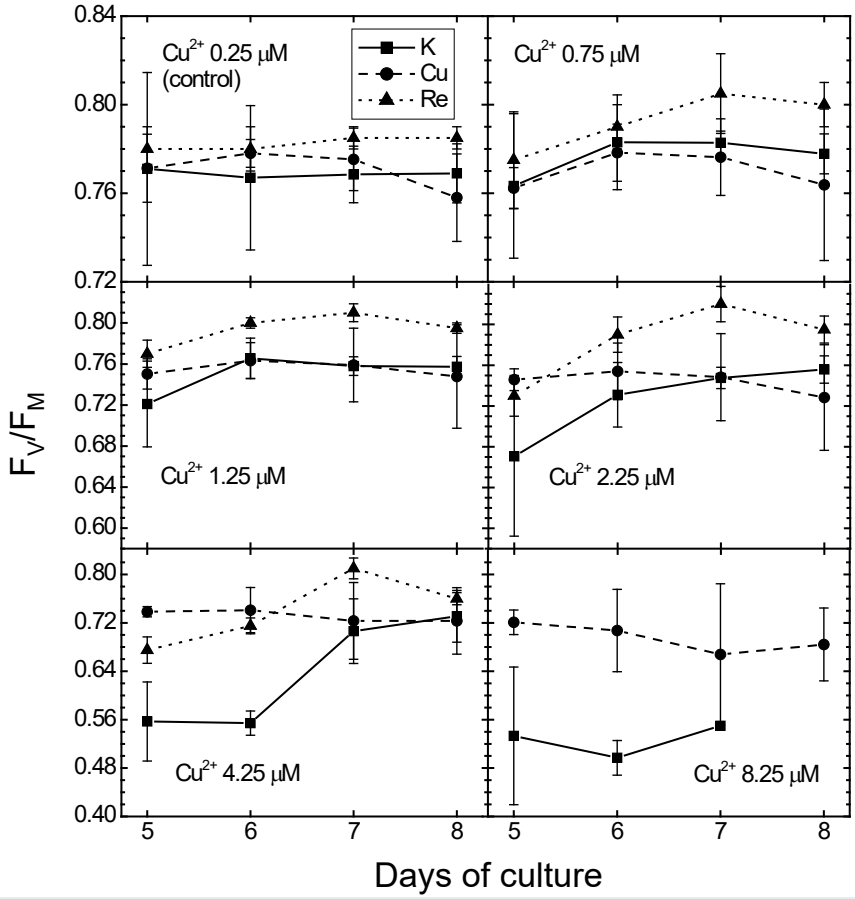

Fig. 3 Changes of maximal quantum efficiency of photosystem II (F_V/F_M) from fifth to eighth day after inoculation in *Chlamydomonas reinhardtii* CW15 cultures on media with different Cu^{2+} concentration. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population.

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

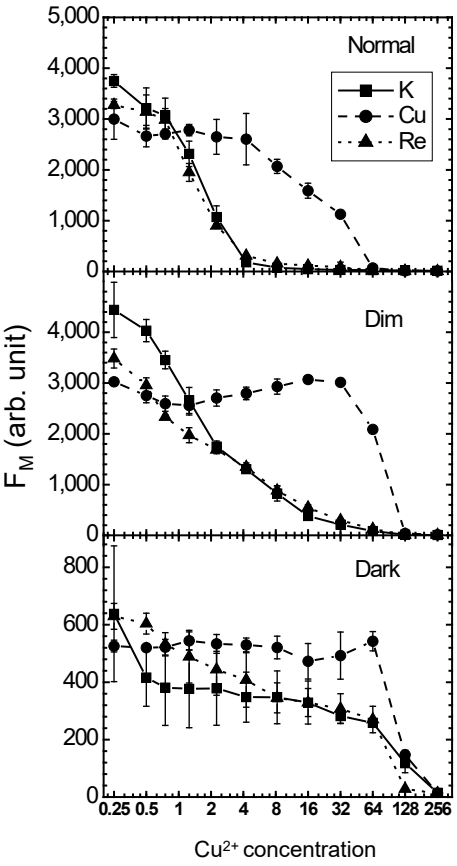

Fig. 4 *Chlamydomonas reinhardtii* CW15 culture densities (measured as F_M, arbitrary units) after 3 days of growth on media with different Cu^{2+} content and under three light regimes: normal – 28 µmol m^{-2} s^{-1} of PAR, continuous light; dim – 6.25 µmol m^{-2} s^{-1} of PAR, continuous light; dark – continuous darkness. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population. Pay attention to the FM scales!

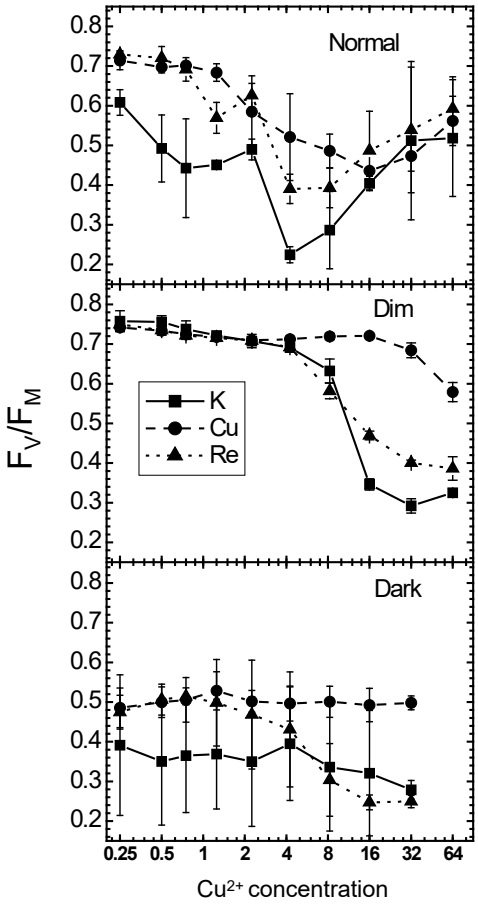

Fig. 5 Maximal quantum efficiency of photosystem II (F_V/F_M) in *Chlamydomonas reinhardtii* CW15 cultures in third day after inoculation on media with different Cu^{2+} concentration and under three light regimes: normal – 28 µmol m^{-2} s^{-1} of PAR, continuous light; dim – 6.25 µmol m^{-2} s^{-1} 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		log(1 − F_V/F_M)	
	F_{df}	p	F_{df}	p
Light conditions	$108.38_{2, 304}$	<0.0001	$502.89_{2, 245}$	<0.0001
Experimental population	$0.23_{2, 304}$	0.7961	$4.82_{2, 245}$	0.0108
Log Cu concentration	$775.43_{1, 304}$	<0.0001	$140.26_{1, 245}$	<0.0001
Log Cu Concentration × Experimental Population	$4.60_{2, 304}$	0.0107	$20.26_{2, 245}$	<0.0001
Experimental Population × Light Conditions	$11.35_{4, 304}$	<0.0001	$17.07_{4, 245}$	<0.0001
Log Cu Concentration × Light Conditions	$62.59_{2, 304}$	<0.0001	$56.23_{2, 245}$	<0.0001
Experimental Population × Light Conditions × Log Cu Concentration			$12.28_{4, 245}$	<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 Cu^{2+} (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 Cu^{2+} 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 Cu^{2+} concentration. The highest and most stable growth over the wide range of Cu^{2+} concentrations (0.25–64 μM Cu^{2+}) 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 Cu^{2+} concentrations lower than 1.25 μM, the Cu culture densities were below those of the K and Re populations.

Tab. 3 Separate analyses for the tree light conditions. Algae growth (log F_M) and algae photosynthetic activity [log(1 – F_V/F_M)] were analyzed in relation to experimental population, log Cu concentration, and their interaction.

	Experimental population		Log Cu concentration		Experimental Population × Log Cu Concentration	
Light condition	F_{df}	p	F_{df}	p	F_{df}	p
			log F_M			
Normal	$5.71_{2, 98}$	0.0045	$487.72_{1, 98}$	<0.0001		
Dim	$1.10_{2, 96}$	0.3379	$267.55_{1, 96}$	<0.0001	$5.05_{2, 96}$	0.0082
Dark	$12.75_{2, 98}$	<0.0001	$83.01_{1, 98}$	<0.0001		
			log(1 – F_V/F_M)			
Normal	$27.10_{2, 77}$	<0.0001	$38.68_{1, 77}$	<0.0001	$4.78_{2, 77}$	0.0110
Dim	$5.53_{2, 78}$	0.0057	$327.89_{1, 78}$	<0.0001	$62.23_{2, 78}$	<0.0001
Dark	$0.07_{2, 80}$	0.9322	$4.41_{1, 80}$	0.0390		

The photosynthetic activity (F_V/F_M) 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 Cu^{2+} (>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 F_V/F_M 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 Cu^{2+} 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 F_V/F_M. In contrast, in the K population, there was strong and progressive inhibition within the whole range of Cu^{2+} concentrations used. High variation of the F_V/F_M parameter in darkness (Fig. 5) is the likely reason why the interaction between the experimental population and Cu^{2+} 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 F_V/F_M in copper-adapted and control population of CW15 to cadmium and zinc ions excess.

Variation in algae growth measured in relation to Cd^{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^{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^{2+} adaptation experiments against various Cd^{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_V/F_M was shaped by three two-way interactions of the main factors (Tab. 4). Analyses performed separately for each Cd^{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^{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^{2+} concentration (Fig. 8). The other populations showed

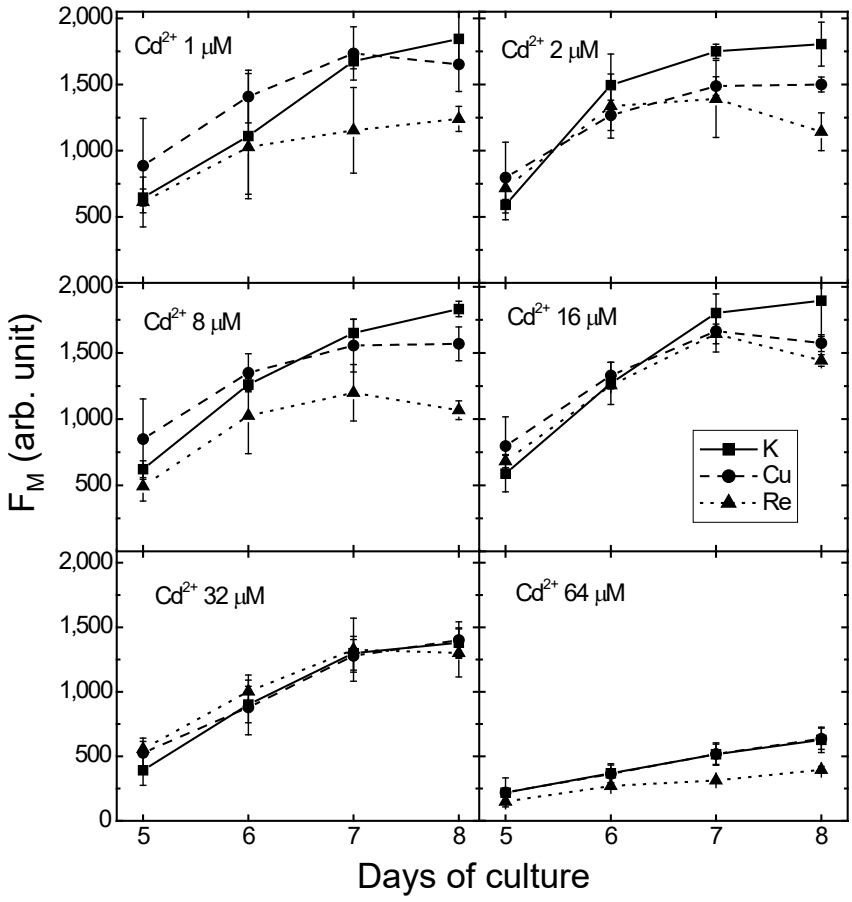

Fig. 6 Growth of *Chlamydomonas reinhardtii* CW15 cultures on media with different Cd^{2+} concentration, measured as F_M. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population. The control without Cd^{2+} – see Fig. 2, 0.25 µM Cu^{2+}.

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_{df}	p	F_{df}	p
Experimental population	$3.72_{2, 336}$	0.0253	$6.04_{2, 334}$	0.0026
Log Cd concentration	$24.71_{1, 336}$	<0.0001	$48.66_{1, 334}$	<0.0001
Time	$39.31_{1, 336}$	<0.0001	$0.03_{1, 334}$	0.8683
Log Cd Concentration × Time	$6.62_{1, 336}$	0.0105	$18.44_{1, 334}$	<0.0001
Experimental Population × Time	$4.44_{2, 336}$	0.0125	$5.36_{2, 334}$	0.0051
Experimental Population × Log Cd Concentration			$73.03_{2, 334}$	<0.0001

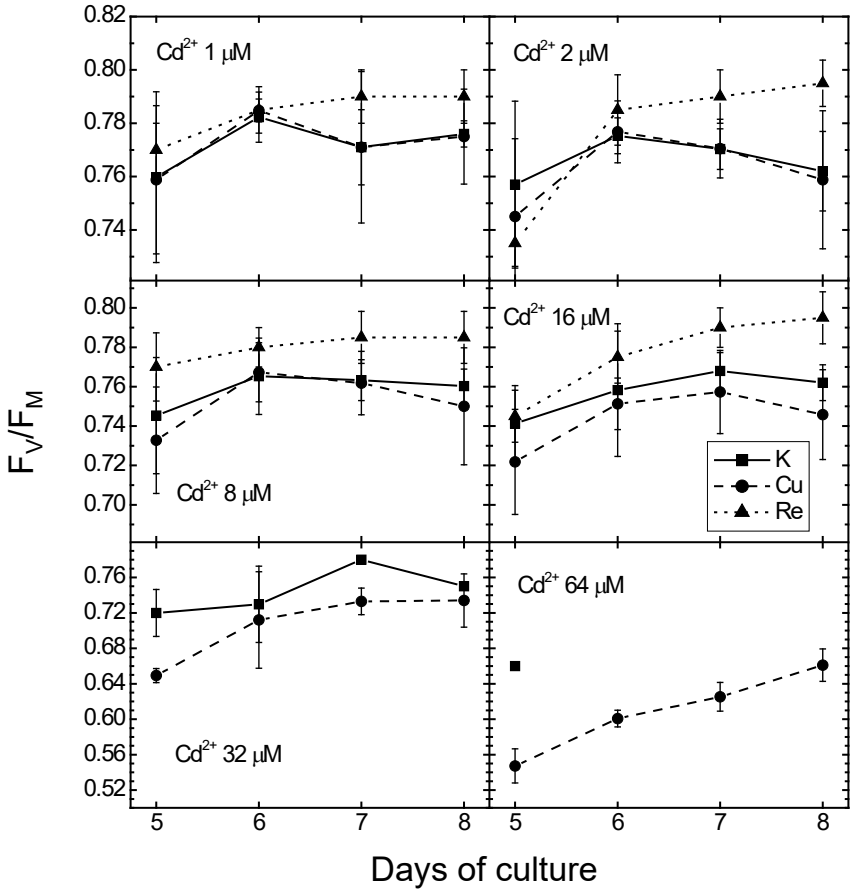

Fig. 7 Changes of maximal quantum efficiency of photosystem II (F_V/F_M) from fifth to eight day after inoculation in *Chlamydomonas reinhardtii* CW15 cultures on media with different Cd^{2+} concentration. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population. The control 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	$1.89_{2, 65}$	0.1588	$0.57_{2, 75}$	0.5673
Zn concentration	$7.68_{1, 65}$	0.0073	$16.06_{1, 72}$	0.0001
Time	$34.97_{1, 65}$	<0.0001	$2.62_{1, 72}$	0.1102
Experimental Population × Zn Concentration	$5.34_{2, 65}$	0.0071	$6.19_{2, 72}$	0.0033
Zn Concentration × Time	$4.68_{2, 65}$	0.0342	$6.19_{1, 72}$	0.0152

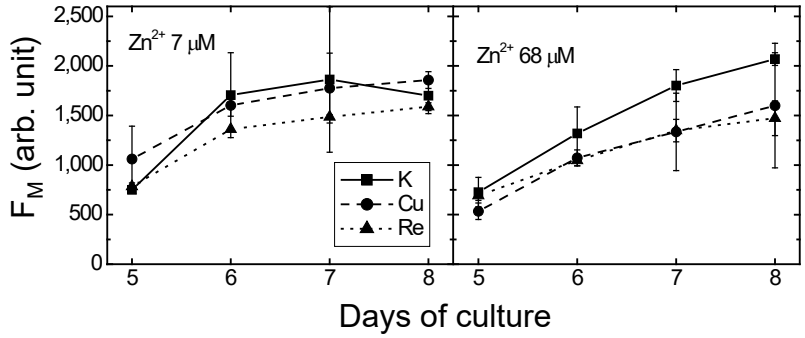

Fig. 8 Growth of *Chlamydomonas reinhardtii* CW15 cultures on media with elevated Zn^{2+} concentration, measured as F_M. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population. The control with standard Zn^{2+} concentration – see Fig. 2, 0.25 µM Cu^{2+}.

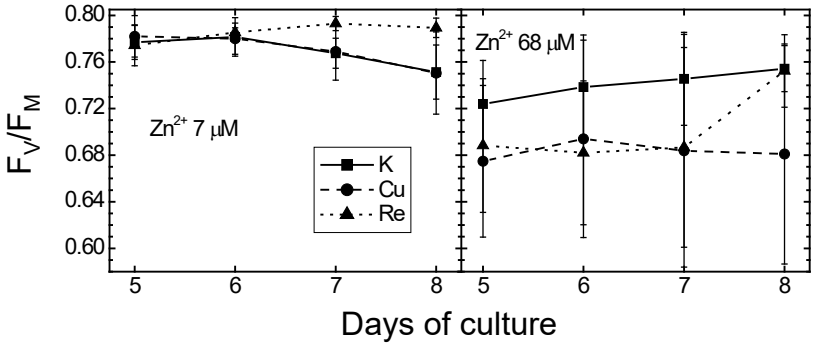

Fig. 9 Changes of maximal quantum efficiency of photosystem II (F_V/F_M) from fifth to eight day after inoculation in *Chlamydomonas reinhardtii* CW15 cultures on media with elevated Zn^{2+} concentration. K – control population; Cu – Cu^{2+}-adapted population; Re – revertant population. The control 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 Cu^{2+} 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 Cd^{2+} concentrations. Tolerant cells were able to multiply even at 300 μM Cd^{2+} (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 Cu^{2+} 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 Cu^{2+}. 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 Cu^{2+} 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 Cd^{2+} 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 Cd^{2+}, 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.*

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 Cu^{2+} 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 (F_V/F_M) of the Cu population, whereas it was strongly inhibited at that concentration in the control population (Fig. 3). The F_V/F_M in the Re population showed only slightly lower resistance than Cu at 4.25 μM of Cu^{2+}, 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 b_{559} 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 Cu^{2+}) when light intensity was increased from 100 to 150 W m^{-2} in *Chlorella vulgaris* cultures. The decrease of Cu^{2+} 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 Cu^{2+} 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 Cu^{2+} because the adapted Cu population does not show any increased resistance to Cd^{2+} or Zn^{2+} (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 Zn^{2+}, as reported by Lin and Aarts [59].

Cu^{2+} 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 antioxidant 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 Cd^{2+} and Zn^{2+}. 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.

Acknowledgments

The Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education.

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.

References

1. Nagajyoti PC, Lee KD, Sreekanth TVM. Heavy metals, occurrence and toxicity for plants: a review. Environ Chem Lett. 2010;8:199–216. https://doi.org/10.1007/s10311-010-0297-8
2. Marschner H. Mineral nutrition of higher plants. London: Academic Press; 1995.
3. Holm RH, Kennepohl P, Solomon EI. Structural and functional aspects of metal sites in biology. Chem Rev. 1996;96(7):2239–2314. https://doi.org/10.1021/cr9500390
4. Festa RA, Thiele DJ. Copper: an essential metal in biology. Curr Biol. 2011;21:877–883. https://doi.org/10.1016/j.cub.2011.09.040
5. Quinn JM, Eriksson M, Moseley JL, Merchant S. Oxygen deficiency responsive gene expression in *Chlamydomonas reinhardtii* through a copper-sensing signal transduction pathway. Plant Physiol. 2002;128:463–471. https://doi.org/10.1104/pp.010694
6. Palmer CM, Guerinot ML. Facing the challenges of Cu, Fe and Zn homeostasis in plants. Nat Chem Biol. 2009;5:333–340. https://doi.org/10.1038/nchembio.166
7. Burkhead JL, Reynolds KAG, Abdel-Ghany SE, Cohu CM, Pilon M. Copper homeostasis. New Phytol. 2009;182:799–816. https://doi.org/10.1111/j.1469-8137.2009.02846.x
8. Pinto E, Sigaud-kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P. Heavy metal-induced oxidative stress in algae. J Phycol. 2003;39:1008–1018. https://doi.org/10.1111/j.0022-3646.2003.02-193.x
9. Mallick N. Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: response of the antioxidant system. J Plant Physiol. 2004;161:591–597. https://doi.org/10.1078/0176-1617-01230
10. Yruela I. Copper in plants: acquisition, transport and interactions. Funct Plant Biol. 2009;36:409–430. https://doi.org/10.1071/FP08288
11. Lu CM, Zhang JH. Copper-induced inhibition of PSII photochemistry in cyanobacterium *Spirulina platensis* is stimulated by light. J Plant Physiol. 1999;154:173–178. https://doi.org/10.1016/S0176-1617(99)80206-5
12. Knauert S, Knauer K. The role of reactive oxygen species in copper toxicity to two freshwater green algae. J Phycol. 2008;44:311–319. https://doi.org/10.1111/j.1529-8817.2008.00471.x
13. Stanley RA. Toxicity of heavy metals and salts to Eurasian watermilfoil (*Myriophyllum spicatum* L.). Arch Environ Contam Toxicol. 1974;2:331–341. https://doi.org/10.1007/BF02047098
14. Wong MH, Bradshaw AD. Comparison of the toxicity of heavy metals, using root elongation of rye grass, *Lolium perenne*. New Phytol. 1982;91:255–261. https://doi.org/10.1111/j.1469-8137.1982.tb03310.x
15. Ince NH, Dirilgen N, Apikyan IG, Tezcanli G, Üstün B. Assessment of toxic interactions of heavy metals in binary mixtures: a statistical approach. Arch Environ Contam Toxicol. 1999;36:365–372. https://doi.org/10.1007/PL00006607
16. Macfie SM, Tarmohamed Y, Welbourn PM. Effects of cadmium, cobalt, copper, and nickel on growth of the green alga *Chlamydomonas reinhardtii*: the influences of the cell wall and pH. Arch Environ Contam Toxicol. 1994;24:454–458. https://doi.org/10.1007/BF00214835
17. Macfie SM, Welbourn PM. The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Arch Environ Contam Toxicol. 2000;39:413–419. https://doi.org/10.1007/s002440010122
18. Mallick N, Rai LC. Physiological responses of non-vascular plants to heavy metals. In: Prasad MNV, Strzałka K, editors. Physiology and biochemistry of metal toxicity and tolerance in plants. Dordrecht: Springer; 2002. p. 111–147. https://doi.org/10.1007/978-94-017-2660-3_5
19. Lindberg S, Greger M. Plant genotypic differences under metal deficient and enriched conditions. In: Prasad MNV, Strzałka K, editors. Physiology and biochemistry of metal toxicity and tolerance in plants. Dordrecht: Springer; 2002. p. 357–393. https://doi.org/10.1007/978-94-017-2660-3_14
20. Hanikenne M. *Chlamydomonas reinhardtii* as a eukaryotic photosynthetic model for studies of heavy metal homeostasis and tolerance. New Phytol. 2003;159:331–340. https://doi.org/10.1046/j.1469-8137.2003.00788.x
21. Merchant S, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, et al. The

Chlamydomonas genome reveals the evolution of key animal and plant functions. Science. 2007;318:245–250. https://doi.org/10.1126/science.1143609

22. Luis P, Behnke K, Toepel J, Wilhelm C. Parallel analysis of transcript levels and physiological key parameters allows the identification of stress phase gene markers in *Chlamydomonas reinhardtii* under copper excess. Plant Cell Environ. 2006;29:2043–2054. https://doi.org/10.1111/j.1365-3040.2006.01579.x
23. Jamers A, Blust R, de Coen W, Griffin JL, Jones OAH. Copper toxicity in the microalga *Chlamydomonas reinhardtii*: an integrated approach. Biometals. 2013;26:731–740. https://doi.org/10.1007/s10534-013-9648-9
24. Prasad MNV, Drej K, Skawińska A, Strzałka K. Toxicity of cadmium and copper in *Chlamydomonas reinhardtii* wild-type (WT 2137) and cell wall deficient mutant strain (CW 15). Bull Environ Contam Toxicol. 1998;60:306–311. https://doi.org/10.1007/s001289900626
25. Fujiwara S, Kobayashi I, Hoshino S, Kaise T, Shimogawara K, Usuda H, et al. Isolation and characterization of arsenate-sensitive and resistant mutants of *Chlamydomonas reinhardtii*. Plant Cell Physiol. 2000;41:77–83. https://doi.org/10.1093/pcp/41.1.77
26. Collard JM, Matagne RF. Isolation and genetic analysis of *Chlamydomonas reinhardtii* strains resistant to cadmium. Appl Environ Microbiol. 1990;56:2051–2055.
27. Collard JM, Matagne RF. Cd^{2+} resistance in wild-type and mutant strains of *Chlamydomonas reinhardtii*. Environ Exp Bot. 1994;34:235–244. https://doi.org/10.1016/0098-8472(94)90044-2
28. Nagel K, Voigt J. In vitro evolution and preliminary characterization of a cadmium-resistant population of *Chlamydomonas reinhardtii*. Appl Environ Microbiol. 1989;55:526–528.
29. Davies DR, Plaskitt A. Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardi*. Genet Res. 1971;17:33–43. https://doi.org/10.1017/S0016672300012015
30. Harris EH. The *Chlamydomonas* sourcebook. Amsterdam: Elsevier; 2009.
31. Lichtenthaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 1987;148:350–382. https://doi.org/10.1016/0076-6879(87)48036-1
32. Quinn GP, Keough MJ. Experimental design and data analysis for biologists. Cambridge: Cambridge University Press; 2002. https://doi.org/10.1017/CBO9780511806384
33. Elena SF, Lenski RE. Microbial genetics: evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat Rev Genet. 2003;4:457–469. https://doi.org/10.1038/nrg1088
34. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC. Experimental evolution. Trends Ecol Evol. 2012;27:547–560. https://doi.org/10.1016/j.tree.2012.06.001
35. Devars S, Hernández R, Moreno-Sánchez R. Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by preexposure to mercury or cadmium. Arch Environ Contam Toxicol. 1998;34:128–135. https://doi.org/10.1007/s002449900296
36. Garcıa-Villada L, Rico M, Altamirano M, Sánchez-Martın L, López-Rodas V, Costas E. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: characterisation and future implications in the use of copper sulphate as algaecide. Water Res. 2004;38:2207–2213. https://doi.org/10.1016/j.watres.2004.01.036
37. Correa JA, Gonzalez P, Sanchez P, Munoz J, Orellana MC. Copper-algae interactions: inheritance or adaptation? Environmental Monitoring and Assesment. 1996;40(1):41–54. https://doi.org/10.1007/BF00395166
38. Nielsen HD, Brownlee C, Coelho SM, Brown MT. Inter-population differences in inherited copper tolerance involve photosynthetic adaptation and exclusion mechanisms in *Fucus serratus*. New Phytol. 2003;160:157–165. https://doi.org/10.1046/j.1469-8137.2003.00864.x
39. Bell G. Experimental genomics of fitness in yeast. Proc R Soc Lond B Biol Sci. 2010;277(1687):1459–1467. https://doi.org/10.1098/rspb.2009.2099
40. Finnegan EJ. Epialleles – a source of random variation in times of stress. Curr Opin Plant Biol. 2001;5(2):101–106. https://doi.org/10.1016/S1369-5266(02)00233-9
41. Mather, K. Polygenic inheritance and natural selection. Biol Rev. 1943;18:32–64. https://doi.org/10.1111/j.1469-185X.1943.tb00287.x

42. Rebound X, Bell G. Experimental evolution in *Chlamydomonas*. III. Evolution of specialist and generalist types in environments that vary in space and time. Heredity. 1997;78:507–514. https://doi.org/10.1038/hdy.1997.79
43. Lupi FM, Fernandes HML, Sá-Correia I. Increase of copper toxicity to growth of *Chlorella vulgaris* with increase of light intensity. Microb Ecol. 1998;35:193–198. https://doi.org/10.1007/s002489900074
44. Antal TK, Graevskaya EE, Matorin DN, Volgusheva AA, Osipov VA, Krendeleva TE, et al. Assessment of the effects of methylmercury and copper ions on primary processes of photosynthesis in green microalga *Chlamydomonas moewusii* by analysis of the kinetic curves of variable chlorophyll fluorescence. Biophysics. 2009;54:481–485. https://doi.org/10.1134/S0006350909040149
45. Devriese M, Tsakaloudi V, Garbayo I, León R, Vílchez C, Vigar J. Effect of heavy metals on nitrate assimilation in the eukaryotic microalga *Chlamydomonas reinhardtii*. Plant Physiol Biochem. 2001;39:443–448. https://doi.org/10.1016/S0981-9428(01)01257-8
46. Kučera T, Horáková H, Šonská A. Toxic metal ions in photoautotrophic organisms. Photosynthetica. 2008;46:481–489. https://doi.org/10.1007/s11099-008-0083-z
47. Hill KL, Hassett R, Kosman D, Merchant S. Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. Plant Physiol. 1996;112:697–704. https://doi.org/10.1104/pp.112.2.697
48. Mendez-Alvarez S, Leisinger U, Eggen RIL. Adaptive responses in *Chlamydomonas reinhardtii*. International Microbiology. 1999;2:15–22.
49. Contreras-Porcia L, Dennett G, González A, Vergara E, Medina C, Correa JA, et al. Identification of copper-induced genes in the marine alga *Ulva compressa* (Chlorophyta). Mar Biotechnol. 2011;13:544–556. https://doi.org/10.1007/s10126-010-9325-8
50. Shah FUR, Ahmad N, Masood KR, Peralta-Videa JP, Ahmad FD. Heavy metal toxicity in plants. In: Ashraf M, Ozturk M, Ahmad MSA, editors. Plant adaptation and phytoremediation. Heidelberg: Springer; 2010. p. 71–97. https://doi.org/10.1007/978-90-481-9370-7_4
51. Arellano JB, Lázaro JJ, López-Gorgé J, Barón M. The donor side of photosystem II as the copper-inhibitory binding site. Photosynth Res. 1995;45:127–134. https://doi.org/10.1007/BF00032584
52. Burda K, Kruk J, Schmid GH, Strzałka K. Inhibition of oxygen evolution in photosystem II by Cu(II) ions is associated with oxidation of cytochrome b_{559}. Biochem J. 2003;371:597–601. https://doi.org/10.1042/bj20021265
53. Liu S, Dong FQ, Yang CH, Tang CQ, Kuang TY. Reconstitution of photosystem II reaction center with Cu-chlorophyll *a*. J Integr Plant Biol. 2006;48:1330–1337. https://doi.org/10.1111/j.1744-7909.2006.00352.x
54. Nagalakshmi N, Prasad MNV. Copper-induced oxidative stress in *Scenedesmus bijugatus*: protective role of free radical scavengers. Bull Environ Contam Toxicol. 1998;61:623–628. https://doi.org/10.1007/s001289900806
55. Sandmann G. Böger P. Copper-mediated lipid peroxidation processes in photosynthetic membranes. Plant Physiol. 1980;66:797–800. https://doi.org/10.1104/pp.66.5.797
56. Stoiber TL, Shafer MM, Armstrong DE. Differential effects of copper and cadmium exposure on toxicity endpoints and gene expression in *Chlamydomonas reinhardtii*. Environ Toxicol Chem. 2010;29:191–200. https://doi.org/10.1002/etc.6
57. Peltier G, Thibault P. O_2 uptake in the light in *Chlamydomonas*. Plant Physiol. 1985;79:225–230. https://doi.org/10.1104/pp.79.1.225
58. Foyer CH, Noctor G. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant. 2003;119:355–364. https://doi.org/10.1034/j.1399-3054.2003.00223.x
59. Lin YF, Aarts MGM. The molecular mechanism of zinc and cadmium stress response in plants. Cellular and Molecular Life Sciences. 2012;69:3187–3206. https://doi.org/10.1007/s00018-012-1089-z
60. Cuypers A, Keunen E, Bohler S, Jozefczak M, Opdenakker K, Gielen H, et al. Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling. In: Gupta DKG, Sandalios LM, editors. Metal toxicity in plants: perception, signaling and remediation. Berlin: Springer; 2011. p. 65–90.
61. Grzyb J, Waloszek A, Latowski D, Więckowski S. Effect of cadmium on ferredoxin: $NADP^+$ oxidoreductase activity. J Inorg Biochem. 2004;98:1338–1346.

https://doi.org/10.1016/j.jinorgbio.2004.04.004

62. Acan NL, Tezcan EF. Inhibition kinetics of sheep brain glutathione reductase by cadmium ion. Biochem Mol Med. 1995;54:33–37. https://doi.org/10.1006/bmme.1995.1005

63. Volland S, Schaumlöffel D, Dobritzsch D, Krauss GJ, Lütz-Mein U. Identification of phytochelatins in the cadmium-stressed conjugating green alga *Micrasterias denticulata*. Chemosphere. 2013;91:448–454. https://doi.org/10.1016/j.chemosphere.2012.11.064