Mechanisms of detoxification of high copper concentrations by the microalga *Chlorella sorokiniana*

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Abstract

Microalgae have evolved mechanisms to respond to changes in copper ion availability, which are very important for normal cellular function, to tolerate metal pollution of aquatic ecosystems, and for modulation of copper bioavailability and toxicity to other organisms. Knowledge and application of these mechanisms will benefit the use of microalgae in wastewater processing and biomass production, and the use of copper compounds in the suppression of harmful algal blooms. Here, using electron microscopy, synchrotron radiation-based Fourier transform infrared spectroscopy, electron paramagnetic resonance spectroscopy, and X-ray absorption fine structure spectroscopy, we show that the microalga *Chlorella sorokiniana* responds promptly to Cu$^{2+}$ at high non-toxic concentration, by mucilage release, alterations in the architecture of the outer cell wall layer and lipid structures, and polyphosphate accumulation within mucilage matrix. The main route of copper detoxification is by Cu$^{2+}$ coordination to polyphosphates in penta-coordinated geometry. The sequestrated Cu$^{2+}$ was accessible and could be released by extracellular chelating agents. Finally, the reduction of Cu$^{2+}$ to Cu$^{1+}$ appears also to take place. These findings reveal the biochemical basis of the capacity of microalgae to adapt to high external copper concentrations and to serve as both, sinks and pools of environmental copper.
Introduction

Microalgae can be exposed to high copper concentrations, in some cases >100 mg/L [1], in polluted aquatic ecosystems and wastewaters. Copper is a major pollutant that enters bodies of water from industrial and mine effluents, agricultural runoffs, residential expulsions, and anti-fouling paints [2, 3]. Regarding the impact on microalgae, copper is both a known algaeicide and an essential nutrient, depending on the concentration [4]. Because photosynthetic microalgae are primary producers of O₂ and biomass, any negative impact of copper on these microorganisms will affect the function of aquatic ecosystems [5]. On the other hand, it has been well established that microalgae modulate bioavailability and toxicity of copper to other organisms [6, 7]. The interactions with copper are also relevant for the use of microalgae in wastewater processing, as well as for the use of wastewaters for microalgal biomass production that is non-competitive with feed sources [8]. Finally, copper compounds are often applied to suppress harmful algal blooms and there is room for optimization of this approach [9, 10]. Thus, it is important to elucidate the mechanisms of toxicity of copper in microalgae as well as mechanisms of adaptation and tolerance to high concentrations of copper [11, 12].

The reaction of microalgae to high copper concentrations is not fully understood. Mechanisms of copper detoxification may involve copper binding to the cell wall and excreted polymers (mucilage) [6, 13, 14], as well as transport into the cell and sequestration/storage by proteins and other molecules, such as metallothioneins and phytochelatins, either within the cytosol or internal compartments [15, 16]. Copper is a redox-active metal with two forms – Cu²⁺ and Cu¹⁺. In microalgae, copper may form coordinate bonds with phosphates and/or different N and S ligands (in plastocyanin, phytochelatins, glutathione) [15, 17, 18]. Furthermore, excess copper may substitute other essential metals with structural or enzyme co-factor roles, leading to the loss of functions and cellular damage [19].

The aim of this study was to elucidate the mechanisms of copper tolerance in *Chlorella sorokiniana*, a green microalgal strain of high environmental and biotechnological relevance [20]. The response and interactions of copper with microalgae were analyzed using a set of methods: scanning electron
microscopy (SEM) with energy dispersive X-ray spectrometry (EDS), synchrotron radiation-based Fourier transform mid-infrared spectroscopy (SR-FTIR), electron paramagnetic resonance spectroscopy (EPR), and X-ray absorption fine structure (i) spectroscopy - X-ray absorption near edge structure (XANES), and extended X-ray absorption fine structure (EXAFS). These approaches provided an improved molecular-level understanding of copper detoxification processes in freshwater green microalgae.

Experimental procedures

Cell cultivation

*C. sorokiniana* strain CCAP 211/8K (alternative designation UTEX 1230) was obtained from the Culture Collection of Algae and Protozoa, UK. Microalgal inocula were added to 150 mL of 3N-BBM+V medium in 250 mL Erlenmeyer flasks at the initial density of 0.5 × 10⁶ cells/mL. The medium was prepared according to CCAP recipe, with the following composition (per 1 L): 750 mg NaNO₃, 25 mg CaCl₂·2H₂O, 75 mg MgSO₄·7H₂O, 75 mg K₂HPO₄·3H₂O, 175 mg KH₂PO₄, 25 mg NaCl, trace element solution (no copper), and vitamins B₁ and B₁₂ [21]. Initial pH of the medium was ~7.5. Microalgae were grown for 20 days at 22°C on orbital shakers (120 rpm) in growth cabinet with a continuous photon flux of 120 μmol m⁻² s⁻¹ (MST TL-D Reflex 36W840 1 SLV/25 tubes, Philips, Amsterdam, Netherlands). Flasks with samples were weighed on day 0 and the volume of samples was corrected for evaporation at day 15 with sterile deionised water before the treatment. Microalgae were exposed to CuCl₂ at day 20 during stationary phase. The phase was determined by optical density at 750 nm (OD₇₅₀) and biomass measurements. For biomass determination, 2 mL aliquots of cultures were centrifuged at 5000 g for 5 min and supernatant was discarded. Pellet was left to dry for 24 h at 60°C and then weighed. Copper was applied to 150 mL samples at the following nominal (total) concentrations: 0.1, 1, 2, 5, and 10 mM. Untreated microalgae were used as controls. The effects of copper addition on cell viability were tested using the Evans Blue stain, as described previously [22]. Evans Blue is a commonly used measure of cell
viability; non-viable cells show destabilized or ruptured membranes with loose permeability that allows accumulation of Evans Blue dye in the cell. An increased proportion of Evans Blue stained cells in a population indicates an increased proportion of non-viable cells. The viability is presented as % of Evans Blue negative cells. At least 100 cells were analysed per sample. In addition, OD_{750} and biomass were established at 1 h, 1 day, 2 days and 7 days after application of copper at different concentrations. These three parameters were analyzed in eight biological replicates from two separate experiments. All chemicals were acquired from Sigma-Aldrich (St. Louis, MI, USA).

**SEM with EDS**

Microalgal samples aliquots (1.5 mL) were untreated or treated with 1 mM Cu^{2+} (CuCl_2) for 15 min, 1 h or 1 day in 3N-BBM-V, washed once with the medium with no added vitamins, and collected for further analysis. Cells were spun at 5000 g for 5 min. Fixation was performed in the same medium (1.5 mL) with 2.5% glutaraldehyde (Serva, Heidelberg, Germany) for 15 min, followed by 2.5% glutaraldehyde in 60 mM phosphate buffer solution (PBS; pH 7.2; 1.5 mL) for 30 min. After washing the cells three times in 1.5 mL PBS, the samples were dehydrated in a graded ethanol series (50%, 70%, 90% and 100%). Samples were dried in Critical Point Dryer CPD300 (Leica, Wetzlar, Germany), sputter coated with carbon (ACE600 Leica, Wetzlar, Germany), and examined with a SEM (Versa 3D, FEI, Hillsboro, OR, USA). EDS was performed with an Octane Pro silicon drift detector (EDAX/AMTEK, Mahwah, NJ, USA) at 20 kV, and a working distance of 10 mm. Monte Carlo simulation (Casino v2.48; 8 simulation with 1000 electron-sample interaction each) [23] revealed that EDS detected X-ray signals from up to 1 µm into the biomass with a x/y resolution of 2.2 µm. EDS results were presented as atomic fractions, which stand for the percentage of moles of a specific element of the total number of moles in the sample.

**SR-FTIR spectroscopy**

Measurements were carried out at the FTIR spectroscopy station of MIRAS beamline at ALBA synchrotron (Barcelona, Spain). Spectra were collected in transmission mode using an infrared
microscope coupled to a FTIR Hyperion 3000 spectrometer (Bruker, Ettlingen, Germany) by using the 36× Schwarzschild objectives. Spectra were obtained using a liquid nitrogen cooled mercury cadmium telluride detector with the accumulation of 128 scans and spectral resolution of 4 cm\(^{-1}\). The aperture was set to size 20×20 μm. Samples (150 mL) were untreated or treated with 1 mM Cu\(^{2+}\) for 1 h. Cells were spun down and washed 3× with medium (50 mL), freeze dried over night and powdered. Spectra were obtained from seven biological replicates of control and treated microalgae. The fingerprint regions for the main bio-molecular families: carbohydrates and nucleic acids (1480-900 cm\(^{-1}\)), proteins (1790-1480 cm\(^{-1}\)), and lipids (3030-2800 cm\(^{-1}\)), were analysed. In addition, we calculated the ratio of C=O stretching absorbance integrated under the band with the maximum at 1740 cm\(^{-1}\), and the sum of asymmetric stretching CH\(_2\) and CH\(_3\) absorbances integrated under the bands from 2990 cm\(^{-1}\) to 2900 cm\(^{-1}\). The ratio represents a parameter of oxidative stress [24, 25]. It is worth mentioning that SR-FTIR has more than 1000-fold higher brightness than conventional sources, which improves the S/N ratio and spectral stability, thus allowing precise peak detection with spatial resolution that reaches the diffraction limit [26, 27]. All spectra were baseline corrected and vector normalized before they were smoothed with 11 iterations. Second derivative of Amides and esters groups was presented for the region between 1800-1480 cm\(^{-1}\) after smoothing 15 iterations and afterwards vector normalization.

**EPR spectroscopy**

Microalgal cultures (150 mL) were exposed to 1 mM Cu\(^{2+}\) for 1 h, spun down and washed once with the same volume of medium, after which samples were divided into two parts. One part was washed 3x with water (50 mL), and the other was washed 3x with 20 mM ethylenediaminetetraacetic acid (EDTA) solution (50 mL), to remove Cu\(^{2+}\) that is bound to the cell surface. Supernatant was removed, and cells were freeze-dried over night and powdered. Samples (10-15 mg) were placed into quartz tubes. Measurements were performed at room T (293 K) on Bruker EMX Nano X-band (9.65 GHz) spectrometer, using the following settings: power attenuation, 13 dB; modulation amplitude, 0.4 mT; modulation frequency, 100 kHz; sweep time, 120 s. Spectra were normalized to biomass. Spectral
simulations were performed to establish g-values and hyperfine splitting (\(A\)), using Hyperfine Spectrum Software (WF Hagen, TU Delft, The Netherlands) [28].

**XAFS spectroscopy**

XAFS spectroscopy can be applied to original aggregate states and to low concentration samples, so it is of special importance for non-crystalline materials. Cell cultures (150 mL) were untreated or exposed to 1 mM Cu\(^{2+}\) for 1 h. Cells were spun down and washed 3× with medium (50 mL). Samples were freeze dried over night and powdered. XANES spectra of standard copper compounds - CuO, Cu\(_2\)O and Cu foil were also measured on the beam-line. XANES and EXAFS experiments were performed using beamline BL22 at ALBA synchrotron. Focused beam size 500 × 500 µm\(^2\) was used. The spectra of algae were collected in fluorescence mode in energy interval from 8870 to 9600 eV with energy stepping resolution of 0.2 eV. Standards samples of Cu were prepared by mixing a small amount of compounds with boron-nitride. Spectra were collected in transmission mode. The XANES analysis was carried out in the range from 8900 to 9100 eV using ATHENA software package [29]. Averaged data values from three consecutive measurements and 6 detector channels were taken in analysing process. Spectra were normalized by three-order polynomial fit. The first inflection point in the XANES spectrum of Cu-foil at 8979.5 was chosen as an initial value for photoelectron threshold energy (E\(_0\)). Absorption edge energy for each sample was determined precisely from the derivative XANES spectra. Fitting of EXAFS spectra was performed by ARTEMIS software package [29]. The k data space was windowed in the interval from 3 to10.4 Å\(^{-1}\). Data were multiplied by k\(^3\) factor and converted in reciprocal space by Fourier transformation in the range of 1-4.8 Å. The absorber-scatterer distance with less than 0.1 Å deviation from participating paths lengths and Debye-Waller factor below 0.03 Å were obtained.

**Statistics**
All experiments were performed at least in three replicates. Results are presented as mean ± standard error (SE), where appropriate. Statistical significance (p < 0.05) in comparison to controls was calculated using Mann-Whitney U 2-tailed test.

**Results and Discussion**

**Release of mucilage polysaccharides and polyphosphate accumulation in response to copper exposure**

*C. sorokiniana* responded rapidly within minutes to exposure to copper (Figure 1). The release of mucilage was initiated within 15 min of exposure, and it accumulated further during 1 h (Figure 1a). In response to exogenous stress, microalgae may ‘strengthen’ their defence by releasing mucilage, a complex amorphous adhesive matrix that is immersed in the surrounding fluid and that alters the chemical milieu of microalgal cells [30, 31]. *Chlorella* strains have been reported to release a soluble type of mucilage which is predominately (up to 90%) composed of polysaccharides [31]. Mucilage polysaccharides in *C. sorokiniana* are built of: sucrose, galacuronic acid, xylitol, inositol, ribose, mannose, arabinose, galactose, and rhamnose (rank order according to the fraction in biomass) [30]. Furthermore, it has been shown that copper and other metals accumulate in microalgal mucilage [30, 32, 33], and that the mucilage binding of free copper ions suppresses toxicity [34]. It is important to note that copper was applied at a concentration that was at the upper limit of non-toxicity to the *C. sorokiniana* culture, in order to prevent lethality. Namely, 1 mM Cu did not affect the viability and growth parameters of *C. sorokiniana* culture even after 7 days of incubation, whereas higher Cu concentrations up to 10 mM showed negative effects (Figure 2). This implies that the changes that took place in the presence of 1 mM Cu reflect the adaptive response and not cell death/lysis. It is important to point out that concentrations of copper that induced toxic effects in microalgal cultures depend on cell density and on any chelating agent that may be present in the medium (orophosphate in this case) [35]. Next, the impact of copper on the composition of biomass was analysed by EDS (Figure 1b). Atomic fractions changed within 15 min,
which implies that the change is mainly related to the fast release of mucilage. On the other hand, it is important to note that EDS beam penetrated ~1 µm into the samples and that *C. sorokiniana* cells are 3-4 µm in diameter. Therefore, EDS delivered information on elemental composition of mucilage, cell wall, and some intracellular space. In the presence of copper, C and N fractions dropped by approximately 10% and 5%. This is most likely a direct consequence of the drastic increase of fractions of O (+10%; ~2-fold increase), P (+2.5%; less than 0.5% is found in controls), Na (+1%; ~2-fold increase), and Cu (~4% after 15 min of treatment; below limit of detection in controls). The observed increase of atomic fractions of O and P reflects 4:1 ratio in orthophosphate ions. This implies that microalgae accumulated orthophosphate ions from the medium via polymerisation and/or phosphorylation of sugars and proteins. It has been shown that polyphosphates represent the dominant P form in microalgae [15]. Polyphosphates in microalgae can be bound to the cell wall and mucilage and/or they can be stored inside the cells in polyphosphate bodies [15, 36, 37]. In addition, phosphorylated sugars and proteins may be present in mucilage [31]. Microalgae appear to accumulate polyphosphates in response to some other types of stress as well, such as S-deprivation [38]. Further, the increase in atomic fraction of Na, as well as the presence of traces of Ca, Mg, and Fe (not shown), in mucilage and cell wall of microalgae exposed to copper, and not in controls, could be explained by the binding to phosphates. It has been proposed that microalgae use mucilage and polyphosphates as nutrient storage pools and sinks for metallic cations including Cu²⁺ [15, 30, 39]. After 1 day, there was a drop in atomic fraction of Cu (and P), which implies that the process of adaptation was completed at that point [32].

**Molecular composition of *C. sorokiniana* biomass in response to copper exposure**

Changes in the composition of microalgal biomass were further examined using SR-FTIR (Figure 3). This is a well suited method for microalgae investigation that provides information on specific biochemical groups and metal complexes [40, 41]. Figure 3a shows the fingerprint region with predominating absorption of carbohydrates, nucleic acids and phosphates. The most pronounced changes in response to copper took place in the 970-900 cm⁻¹ region. The bands that emerged at ~928 cm⁻¹ and 1123 cm⁻¹ were
assigned to P-O-P asymmetric stretching vibrations in polyphosphates [42, 43]. On the other hand, bands at 1072 cm$^{-1}$ and 1284 cm$^{-1}$, which were assigned to symmetric and asymmetric stretching of P=O in HPO$_4^{2-}$ [44], and the band at 1045 cm$^{-1}$ which may have originated from asymmetric stretching of P=O in H$_2$PO$_4$ [43], showed similar intensities in control and treated microalgae. This implies that the level of free orthophosphate ions was not altered in treated microalgae, and that P was accumulated in polyphosphate form. In line with this, 1233 cm$^{-1}$ and 1249 cm$^{-1}$ bands were stronger and shifted to lower energies in microalgae that were treated with copper. These bands were assigned to polyphosphates and phosphoryl groups on sugars and proteins, respectively [40, 45]. The shift may be explained by the binding of Cu$^{2+}$ to phosphates which results in longer/weaker bonds [41]. A set of absorption bands detected in the spectral region between 1150 and 1000 cm$^{-1}$ come from polysaccharide chains, and were assigned to stretching of C-C, C-O-C, and C-O (in C-OH) [40, 41]. It is important to note that the bands at 1123 and 1224 cm$^{-1}$ may also be related to xanthates and Cu-xanthates (R-O-C=S)-S···Cu$^+$) [45, 46]. The band at 1376 cm$^{-1}$ could be associated with chitosan [47], which is the main component of the outer rigid layer of cell wall in Chlorella [48]. The increase in the intensity of this band implies that cell wall thickening may be a part of the rapid response to copper. Alterations in the cell wall architecture and increased metal binding capacity of the cell wall have been related previously to microalgal defence against high metal concentrations [49]. Likewise, an increase in polysaccharides that has been identified using FTIR and that was indicative of cell wall changes, has been observed in the green microalga Chlamydomonas reinhardtii in response to copper treatment [50].

Next, the exposure to copper led to changes in the protein region (Figure 3b). A broad band at 1586 cm$^{-1}$ (exact wavenumber was obtained from the 2$^{nd}$ derivative), and the band at 1733 cm$^{-1}$ showed increased intensities. On the other hand, the intensities of Amide I 1640 cm$^{-1}$ band and Amide II 1538 cm$^{-1}$ band (C=O H-N stretching and N-H bending vibrations, respectively), were lower in treated microalgae, as confirmed by the 2$^{nd}$ derivative. Decrease in intensities of Amide I and Amide II bands were also observed in Chlamydomonas reinhardtii in response to copper exposure but only in strains that were adapted to copper stress; those that were non-adapted and more sensitive to copper showed stronger
amide bands [50]. A broad band at 1586 cm\(^{-1}\), and the carbonyl groups band at 1733 cm\(^{-1}\) showed increased intensities. The increase of the 1586 cm\(^{-1}\) band that was assigned to NH\(_2\) bending, with parallel decrease of the Amide II band has been reported previously for chitin undergoing partial deacetylation [51, 52]. The decrease of intensity of C=O stretching band at 1640 cm\(^{-1}\) also may occur as a result of deacetylation. Therefore, it appears that N-acetyl-glucosamine moieties in chitosan underwent deacetylation as a part of the adaptation to high copper concentrations. It has been proposed that deacetylation leads to increased solubility of chitosan and increased capacity to bind metals [53, 54]. It is important to note that the band at 1733 cm\(^{-1}\) was assigned to carbonyl groups in proteins and lipids, which accumulate as a result of pronounced oxidation [55]. Finally, a set of changes took place in the lipid region (Figure 3c). The assignation was performed according to available literature [56, 57]. In the spectrum of Cu-treated microalgae, bands that come from asymmetric and symmetric stretching of CH\(_2\) were shifted to higher wavenumbers: 2916→2923 cm\(^{-1}\) and 2851→2855 cm\(^{-1}\) respectively. This has been related to a decrease in conformational order of the acyl chains in lipid membranes and droplets [58, 59]. Bands at 2808, 2997, and 3011 cm\(^{-1}\) were less intensive in treated microalgae. The first band is not usually observed in FTIR spectra of lipids, and has been previously assigned to interactions between fatty acid chains and flavonoids, through C-H-O hydrogen bonds [60, 61]. It is important to note that lower intensity of the ‘flavonoid band’ is in accord with decreased ordering of acyl chains, since flavonoids generally increase order of lipid layers [62]. The band at 2997 cm\(^{-1}\) was assigned to ester groups (-O-CH\(_3\)), whereas the 3011 cm\(^{-1}\) band comes from C=C-H stretching in unsaturated lipids. Therefore, the exposure to copper appears to result in an altered lipid profile. Changes in microalgae lipid profiles, in particular enhanced biosynthesis and accumulation of triacylglycerol within lipid droplets as an internal energy store, is a conserved general response to environmental stresses, including the response to excess metals [63, 64]. Finally, it has been shown that an excess of copper may induce oxidative stress in microalgae [65]. In our settings, copper induced an increase of the parameter of total oxidative stress (i.e. lipid peroxidation), but changes were non-significant (Figure 3d).
Copper speciation during binding to *C. sorokiniana* biomass

Coordination binding of copper was further examined using EPR and synchrotron-based XAFS spectroscopy. EPR spectrum showed that *C. sorokiniana* binds copper in the Cu$^{2+}$ form ($S = 1/2$; Cu$^{1+}$ is EPR inactive with $S = 0$) (Figure 4). EPR signal showed one strong $g_{\perp}$ line and four weak lines coming from hyperfine coupling with $^{63}$Cu/$^{65}$Cu nuclei ($I = 3/2$) along $g_i$. According to spectral shape, broad $g_{\perp}$ line ($\Delta B_{\perp} \approx 9.5$ mT), and $g$-values ($g_i > g_{\perp} > g_{\text{free electron}}$), Cu$^{2+}$ may be in penta-coordinated square-pyramid or in square-planar geometry [66, 67]. The washing of cells with extracellular copper-chelating agent EDTA resulted in decreased intensity of the Cu$^{2+}$ spectrum, which implies that copper was bound to mucilage and/or the cell wall. Spectra before and after EDTA washing were very similar in shape with the same $g$ and hyperfine splitting $A_i$ values. This implicates the presence of one dominant species of coordinated Cu$^{2+}$. It should be mentioned that large Cu$^{2+}$ complexes, such as copper bound to components of dry biomass, generally give similar spectra at room and low T (293 K and 77 K). The relaxation at room T is faster but not enough to seriously broaden the spectrum [28].

The normalized XANES spectra and derivatives of copper in the microalgal biomass, as well as spectra of Cu$^{2+}$, Cu$^{1+}$ and Cu$^{0}$ standards are presented in Figure 5. The copper spectrum of microalgal biomass showed shoulder(s) at positions that were near the energies of 1s-4p$_{xy}$ shake-down transitions of Cu$^{2+}$ and Cu$^{1+}$ - 8984.0 eV (CuO) and 8980.6 eV (Cu$_2$O) [68]. The lack of distinct 1s-4p$_{xy}$ peaks implies that the geometry of copper coordination is different than for the reference compounds (square-planar in CuO, and two-coordinate tetrahedral in Cu$_2$O). The shape of the spectrum is indicative of a distorted octahedral symmetry of Cu$^{2+}$, and four-coordinate distorted tetrahedral geometry of Cu$^{1+}$ [69–71]. In line with this is the flattened white line peak of copper in biomass that is positioned between 1s-4p$_x$ peak energies of Cu$^{1+}$ and Cu$^{2+}$. Additionally, a broad pre-edge hump clearly points to 1s-3d transition, which is typical for Cu$^{2+}$ due to its open-shell structure. Although 1s-3d is a dipole forbidden transition, it takes place because of partial mixing of 4p and half occupied 3d$_{x^2-y^2}$ orbitals in Cu$^{2+}$ with distorted symmetry. The mixing is induced by ligands. The energy of this orbital is lower than the 4p orbital, so the absorption peak emerges.
at energy that is lower than the absorption edge peak [69]. A similar peak cannot be found for Cu^{1+} due to its 3d^{10} closed shell configuration. The derivative spectra identified an edge energy peak/the first inflection point in microalgal biomass at 8981.6 eV (Figure 5b), which further confirms the presence of Cu^{1+}. Close positions of this peak and the edge energy of the Cu^{1+} standard (Cu_{2}O), implicate similar electronegativity of ligand(s), although with different geometries. It has been proposed previously that Cu^{1+} is accumulated inside the cells, and that it is generated by glutathione-mediated Cu^{2+} reduction or by cell surface reductase [72, 73]. Finally, a prominent pre-edge absorption peak at 8976.6 eV (1s-3d transition) and a peak at 8985.8 eV, point out the presence of Cu^{2+} [67]. This is in line with the findings of a previous XANES study on copper speciation in marine microalgae [15].

EXAFS spectra of Cu in microalgal biomass and corresponding fits are shown in Figure 6. The first insight in spectra clearly shows that the scattering amplitude rapidly damps out, indicating low Z atoms as Cu ligands [74]. The most intensive peak is asymmetric and has a shoulder in the low-R region that reflects k-dependent structure in the phase correction function of the EXAFS equation [71, 75]. The proposed environment encompasses a low Z ligands suggesting that the detoxification mechanism does not involve Cu binding to heavy elements [15]. The results further confirm the co-existence of Cu^{1+} and Cu^{2+}, both with O in the first coordination sphere at distances of 1.927 Å and 2.345 Å. It is important to underline the complexity of the examined biological system: presence of two Cu oxidation forms, low Cu concentration, and an apparent absence of crystal structure. The best fit was obtained using parameters for Cu^{2+} that is bound to phosphate ions Cu_{3}(PO_{4})_{2}, and parameters for Cu^{1+} that is bound to O ligands (Cu_{2}O). The calculated coordination number for Cu^{2+} was 4.98 ± 1.20 ligands for absorbing atom. It is important to note that Cu^{2+} in Cu_{3}(PO_{4})_{2} shows two geometries: two Cu^{2+} ions are in irregular pentacoordinated geometry; and one ion is in distorted square-planar coordination [76]. This is all in accord with the EPR results, which implied penta-coordinated square-pyramid geometry or square-planar geometry. Contributing single scattering paths of Cu and P suggested distances for these two atoms at 2.966 Å and 2.983 Å from the absorbing Cu. The results confirm that Cu^{2+} is bound to polyphosphates. This is in line with previous reports showing the co-localization of polyphosphates and copper in
microalgae [39], accumulation of polyphosphates in copper-resistant strains [16], and a decrease in sensitivity to copper in the presence of phosphates [77, 78]. The introduction of S-ligands into the fits did not return meaningful results that were consistent with XANES and EPR findings. This is important to point out since intracellular Cu\(^{1+}\) is commonly sequestrated by S-ligands, such as glutathione.

**Multiple copper tolerance mechanisms in microalgae**

This study indicated copper binding to secreted mucilage and to the cell wall as a fast adaptive response to sub-toxic concentrations of copper for the microalgae *C. sorokiniana*. However, it is important to note that this is just one mechanism out of many that are utilised by microalgae. When the cell wall and exudates are unable to act as a sufficient barrier to prevent copper internalisation, other tolerance mechanisms are likely to be activated. For example, increased free ion concentration of cytosolic Cu\(^{2+}\) will induce production of phytochelatins to provide intracellular copper binding [79]. It has been proposed that microalgae may reduce Cu\(^{2+}\) to Cu\(^{1+}\) which is commonly coordinated by S-ligands (phytochelatins and glutathione) [15]. Of note, although we detected Cu\(^{1+}\) in *C. sorokiniana*, it appears not to be bound to sulfur. Further, some mechanisms will be rapidly transcriptionally or post-transcriptionally induced in response to copper exposure including putative heat shock proteins or antioxidative activities such as superoxide dismutase and ascorbate peroxidase to mitigate the Cu\(^{2+}\) mediated production of hydroxyl radicals [80–82]. Pertinent to this, glutathione represents both, an important antioxidant and Cu\(^{1+}\) binding agent [15], which probably makes its role in copper detoxication in microalgae still elusive. It is worth noting that glutathione complexes with Cu\(^{1+}\) are redox active [83], which relates them more with active metabolism than detoxication/inactivation. It is important to highlight that the experiments in this study were performed in nutrient-rich (including high phosphate) medium that allowed high cell density, and therefore greater surface area for copper binding. High concentration of external phosphate will mitigate metal toxicity such as through formation of copper complexes with orthophosphate in aqueous solution (orthophosphate buffering), and by enhancing cellular accumulation of phosphate and thus allowing larger amounts of polyphosphate to be synthesised.
[84, 85]. In contrast, it has been observed that metal toxicity may be enhanced under phosphate deficient conditions, in part due to higher binding and accumulation of metals including copper [63, 86]. Therefore, the concentration of phosphate represent an important factor in the (regulation of) response of microalgae to high copper.

In summary, in response to copper exposure, C. sorokiniana ‘undertakes’ rapid transformation of its exterior by releasing mucilage with accumulations of phosphates in polymeric form, changing the mechanical and metal-binding properties of the outer layer of the cell wall. Cu had a strong impact on lipids in C. sorokiniana, inducing changes in mechanical properties of supramolecular lipid structures and the overall lipid profile. The main route of copper detoxification/accumulation is by Cu$^{2+}$ coordination to polyphosphates in penta-coordinated square-pyramid geometry, although the reduction to Cu$^{1+}$ appears also to take place. The cell wall and mucilage may have an important role in buffering high external copper concentrations.

**Abbreviations**

EDS, energy dispersive X-ray spectrometry; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance spectroscopy; EXAFS, extended X-ray absorption fine structure; SEM scanning electron microscopy; SR-FTIR, synchrotron radiation-based Fourier transform mid-infrared spectroscopy; XAFS, X-ray absorption fine structure spectroscopy; XANES, X-ray absorption near edge structure.

**Author Contributions**

M.S., S.V., I.S., J.K.P., B.Z., T.D., and M.R.M. designed the research; S.V., J.D.L., and B.Z. performed TEM experiments; T.D., M.D., and M.Ž. performed FTIR and XAFS experiments and analyzed the data. I.S., S.V., and M.D. performed EPR experiments. All authors participated in the writing of the manuscript. J.K.P., I.S., and B.Z. were involved in overall supervision of the project.

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Conflict of Interest

The authors declare that there are no competing interests associated with the article.
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FIGURE LEGENDS

**Figure 1.** SEM and EDS analysis of *C. sorokiniana* cells that were untreated (controls) or treated with Cu$^{2+}$ at nominal concentration of 1 mM for different incubation periods. (a) Micrographs of microalgae. Individual microalgal cells are clearly visible in the control. Mucilage can be observed covering individual cells as ghostly structures and as amorphous matrix between the cells in samples treated with copper. White circles and boxes indicate the areas in which EDS data were collected. Bars = 5 µm. (b) Atomic fraction of different elements in microalgal biomass (means ± SE), as determined by EDS.

**Figure 2.** Viability, growth and biomass production of *C. sorokiniana* culture during 7 days of exposure to different nominal concentrations of copper. (a) Viability, as determined by Evans blue protocol; (b) Growth, as determined by optical density at 750 nm (OD$_{750}$); (c) Biomass. Culture was exposed to copper in the stationary phase. Data are presented as means ± SE.

**Figure 3.** SR-FTIR spectra of biomass of *C. sorokiniana* culture that was untreated (control) or treated with Cu$^{2+}$ at nominal concentration of 1 mM for 1 h and washed. The fingerprint regions represent: (a) Carbohydrates and nucleic acids, *i.e.* phosphates; (b) Proteins and carbonyl groups; arrowheads mark the position of bands of interest in the 2nd derivative. (c) Lipids. Positive and negative shifts in band positions for Cu-treated algae are presented in brackets. (d) Oxidative stress parameter - the ratio of C=O absorbance (band at 1733 cm$^{-1}$) and the sum of asymmetric CH$_2$ and CH$_3$ absorbances (bands at 2920 and 2957 cm$^{-1}$). Boxes represent the median and the 25th and 75th percentiles; whiskers represent the non-outlier range. Data are also numerically presented as means ± SE. The differences were not statistically significant.

**Figure 4.** Room T (293 K) EPR spectra of biomass of *C. sorokiniana* culture that was untreated (control) or exposed to Cu$^{2+}$ at nominal concentration of 1 mM for 1 h. Prior to freeze-drying, cells were washed...
with H$_2$O (to remove Cu$^{2+}$ from the solution) or EDTA (to remove Cu$^{2+}$ from the cell surface). Recording parameters were: power attenuation, 13 dB; modulation amplitude, 0.4 mT. Gray lines – simulations that delivered the presented g and A values. Peak with $g = 2$ is assigned to an organic radical.

**Figure 5.** XANES spectra of biomass of *C. sorokiniana* culture that was exposed to copper. (a) XANES spectra. Microalgae were exposed to Cu$^{2+}$ at nominal concentration of 1 mM for 1 h and washed. Spectra of Cu$^{2+}$ and Cu$^{1+}$ compounds as well as metallic copper (Cu$^{0}$) are presented for reference. Arrowheads: black – pre-edge peak; white – edge peak(s); gray – ‘white line’ peak. (b) Spectra derivatives. Dashed lines mark characteristic peaks of Cu in the biomass. Box – part of spectra derivatives for algae and CuO in the 8975-8979 eV range.

**Figure 6.** Copper EXAFS data for biomass of *C. sorokiniana* culture that was exposed to Cu$^{2+}$ at nominal concentration of 1 mM. (a) Fourier transformed EXAFS spectrum of algae that were exposed to 1 mM Cu$^{2+}$ for 1 h and washed. (b) $k^3$-weighted Cu K-edge EXAFS spectrum.
