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Mechanisms of sublethal copper toxicity damage to the photosynthetic apparatus of *Rhodospirillum rubrum*☆

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ABSTRACT

Magnesium (Mg^{2+}) is the ubiquitous metal ion present in chlorophyll and bacteriochlorophyll (BChl), involved in photosystems in photosynthetic organisms. In the present study we investigated targets of toxic copper binding to the photosynthetic apparatus of the anoxygenic purple bacterium *Rhodospirillum rubrum*. This was done by a combination of *in vivo* measurements of flash photolysis and fast fluorescence kinetics combined with the analysis of metal binding to pigments and pigment-protein complexes isolated from Cu-stressed cells by HPLC-ICPMS (ICP-sfMS). This work concludes that *R. rubrum* is highly sensitive to Cu^{2+}, with a strong inhibition of the photosynthetic reaction centres (RCs) already at 2 μM Cu^{2+}. The inhibition of growth and of RC activity was related to the formation of Cu-containing BChl degradation products that occurred much more in the RC than in LH1. These results suggest that the shift of metal centres in BChl from Mg^{2+} to Cu^{2+} can occur *in vivo* in the RCs of *R. rubrum* under environmentally realistic Cu^{2+} concentrations, leading to a strong inhibition of the function of these RCs.

1. Introduction

Heavy metals, such as Cu, Zn, Ni and Mn, are essential trace elements for photosynthetic organisms. At very low concentrations, ions of such metals are indispensable components of nutrient media in which these organisms grow. However, in higher concentrations, these metals have severe toxic effects [1,2].

Among the mechanisms proposed to contribute to heavy metal damage we can find inhibition of photosynthesis, which was studied *in vivo* (with higher plants and various types of algae) under environmentally relevant concentrations. One mechanism of inhibition of photosynthesis that was shown to occur at environmentally relevant (sub-micromolar to low micromolar range) concentrations *e.g.* of copper is the substitution of Mg^{2+} in the chlorophyll molecule by those trace metal ions. This reaction leads to the formation of heavy metals substituted chlorophylls ([hms]-Chls), which are unsuitable for photosynthesis for several reasons [3]. Among these reasons, likely the most important are:

1) The very short lifetime of the singlet excited state. As a result, [hms]-Chls have remarkably lower *in vitro* fluorescence quantum yields in comparison with [Mg]-Chl or do not exhibit fluorescence at all (*e.g.* [Cu]-Chl, [Ni]-Chl) [4]. Since the resonance energy transfer from the antenna pigment complexes in the chloroplasts depends on the same excitation states that cause Chl fluorescence, [Cu]-Chl and [Ni]-Chl are not adapted to light harvesting [5]. Consequently, the

☆ NJP did most of the experimental work. HK made the original plans for this work and supervised the project. DK did the *in vivo* fluorescence kinetic measurements; DB did the analysis of RC activity. HK and SNHB designed the HPLC running parameters and coupling to ICP-MS. NJP and HK measured, analysed and interpreted the other data. DKSS started the cultivation and in transferred the LH1/RC isolation protocol from the description in literature to our conditions. NJP and HK wrote the original draft of the manuscript, all authors contributed to further revisions of the manuscript.

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conversion of a few percent of the total Chl to such [hms]-Chls (or BChl to [hms]-BChls) dramatically decreases fluorescence [6–8] and leads to inactivation of the photosynthetic system.

2) [Mg]-Chl a has higher ability of electron release from the singlet excited state than all [hms]-Chl, being Mg^{2+} the most favourable ion for the special-pair Chls in the reaction centres (RCs) [9].
3) Among all metallochlorophylls, the complex with Mg^{2+} has the highest tendency to bind axial ligands, while Cu^{2+} binds none [10]. These axial ligands are essential for the correct binding of the pigment [11] and for the correct folding of the pigment-protein complexes [12].

All the *in vivo* studies on [hms]-Chl formation as a mechanism of photosynthesis inhibition so far, however, have been carried out in organisms that have chlorophylls as pigments, while no organism that has bacteriochlorophyll (BChl) has been investigated in that respect. Only one of the studies on the level of isolated proteins, carried out by Fiedor et al. [8], has shown that the presence of [hms]-BChls in a photosynthetic system has such consequences as well. In how far such a substitution of the central ion in BChl actually occurs under toxicity stress *in vivo* remained unknown. Instead, a very interesting case was discovered where a [hms]-BChl is formed in a controlled and then functional way, this is the [Zn]-BChl in *Acidiphilium*, a group of bacteria that lives in very metal-rich highly acidic waters [13]. Chemically, it is clear that the Mg-substitution in BChl is less favoured than in Chl because Mg^{2+} is much more strongly bound in BChl [14]. Nevertheless, kinetically and thermodynamically [Cu]-BChl is much more stable than BChl towards demetallation. It was hypothesized that Zn^{2+} ions favour chelation under low pH conditions due to their low ionization energy. The strong binding of the Zn^{2+} ion makes it furthermore highly resistant to unwanted transmetallation of [Zn]-BChl to other metal ions such as Cu^{2+} and Hg^{2+} [15]. Therefore, it was hypothesized that the unique formation of [Zn]-BChl in these bacteria is a strategy to prevent a detrimental demetallation or transmetallation in the harsh environments where these bacteria live [3]. But so far, these hypotheses were never experimentally tested.

Rhodospirillum rubrum (Esmarch 1887) Molisch 1907 [16,17] belongs to the genus *Rhodospirillum*, which is the type genus of the family *Rhodospirillaceae* in the class *Alphaproteobacteria*. It is an anoxygenic phototroph that produces extracellular elemental sulfur (instead of oxygen) while harvesting light. Like *Acidiphilium*, it contains one of the simplest photosynthetic systems currently known, lacking light harvesting complex 2 [18]. But contrary to *Acidiphilium*, it contains [Mg]-BChl, so that it should be vulnerable to Mg-substitution if this occurs at all in BChl under environmentally relevant conditions. In stagnant freshwater ecosystems, in which *R. rubrum* grows, copper toxicity most often occurs as result of human activities, such as industry and agriculture. While industrial contamination has drastically decreased due to better industrial practices and wastewater treatment, the agricultural source of copper contamination (the use of copper containing pesticides, especially in vineyards) remains and leads to contaminations up to the low micromolar range [1]. Nevertheless, contrary to higher plants and algae, the mechanism of copper toxicity in purple bacteria under environmentally relevant copper concentrations and anoxic conditions remained largely unknown. Liotenberg et al. [19] investigated only very high copper concentrations (50–200 µM) applied for forcing effects in just 24 h of treatment, used oxic conditions and did not measure photosynthesis. Panwichian et al. [20] isolated Cu-resistant purple bacteria from ponds where the water contained up to 30 ppb (approx. 0.5 µM) of Cu^{2+}, but they did not investigate mechanisms of Cu-stress or Cu-tolerance. Bird et al. [21] worked with environmental copper levels as well, but again did not measure photosynthetic parameters.

In the current study we directly tested the hypothesis that the shift of metal centres in BChl from Mg^{2+} to Cu^{2+} in *R. rubrum* occurs *in vivo* at environmentally relevant copper concentrations and is directly related to the inhibition of photosynthesis during Cu^{2+} toxicity stress.

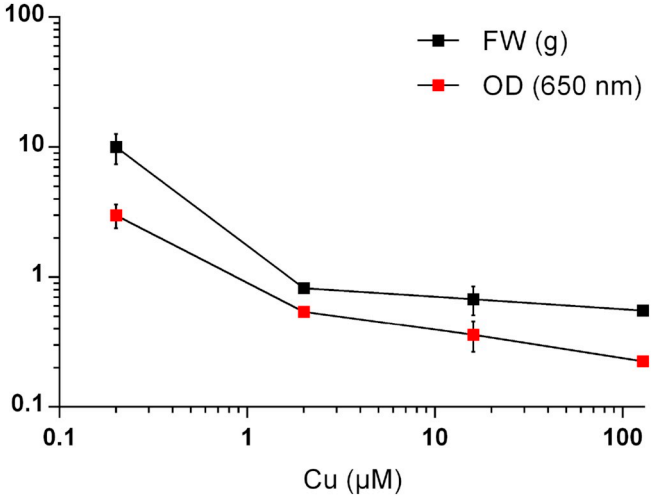

Fig. 1. Effects of Cu on growth, measured as fresh weight and as $OD_{650\ nm}$ of *Rhodospirillum rubrum* cells after four days of treatment with 0.2 (control), 2, 16 and 128 µM of $CuSO_4$. Each value is the mean of 3 replications (3 different experiments, n = 3). Bars represent ± SEM.

2. Material and methods

2.1. Organisms and culture conditions

Rhodospirillum rubrum cells were batch-cultured in 4 L bottles with a defined medium [22], anaerobically gassed with a mixture of 1% CO_2:99% N_2 (traces of oxygen entered through the walls of the tubing), at 30 °C. All experiments were carried out under artificial radiation sources (cool white Osram Dulux L 55 W/840 fluorescent tubes) with a 12:12 h light:dark rhythm. During the light phase, a sinusoidal light curve with a maximum light intensity of 530 µmol·m^{-2}·s^{-1} (PAR) at noon was applied to simulate the diel intensity variation in the environment. The light spectrum mostly excited the B_x and Q_x absorption peaks of BChl, plus the carotenoids. The bacteria were inoculated at an initial OD_{650} of 0.2 in each bottle, from a mother culture at the beginning of its exponential phase of growth (OD_{650} of 4.0–5.0) and treated with four different copper concentrations: 0.2 (control), 2, 16 and 128 µM in the defined medium. Copper was added as $CuSO_4$. The cells were collected after 4 days (exponential phase in the control bottle) by centrifuging at 6800 ×*g* for 5 min. After harvest, the cells were kept at −80 °C. The experiment was repeated six times, but not all measurements could be performed on all repeats. Therefore, the number of repeats for each type of measurement is written in the figures.

2.2. Isolation of light-harvesting 1 (LH1) and reaction centre (RC) complexes

LH1 and RC were isolated from the harvested cells kept at −80 °C. The isolation of complexes was performed using the method described by Picorel et al. [23] with some modifications. Briefly, 0.6 to 1.0 g of frozen collected cells were suspended at 4 °C in 6 mL of 50 mM phosphate buffer (pH 7.0) and sonicated 3 times for 10 min. After each sonication, the samples were centrifuged (10 min at 8000 ×*g*), collecting the supernatant and resuspending the pellet in 6 mL of 50 mM phosphate buffer (pH 7.0). The supernatant was then ultra-centrifuged at 105,000 ×*g* at 4 °C for 1.5 h, discarding the new supernatant obtained and suspending the pellet (chromatophores) for 1 h at 4 °C in 50 mM phosphate buffer (pH 7.0) and 0.5% w/v *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) to a final OD_{880} of 37.5. The LDAO concentration was

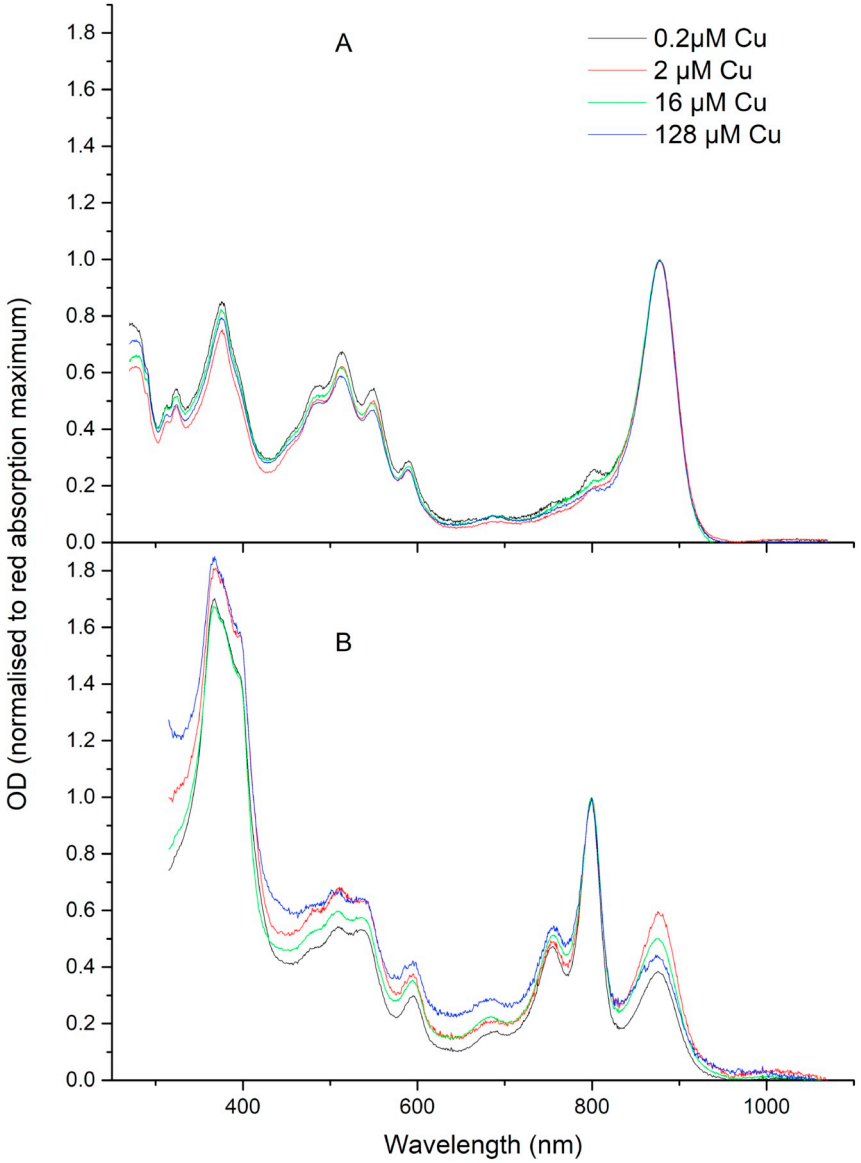

Fig. 2. UV/VIS/NIR absorbance spectra of isolated pigment-protein complexes from *Rhodospirillum rubrum* cells stressed with different Cu^{2+} concentration. The spectra represent averages of two independent experiments. In this and all following figures, the upper graph has the same x-axis as the lower. For better clarity of the graph it is not labelled separately. **A**) light-harvesting complexes (holochromes B880 or LH1); **B**) reaction centres (RCs).

then brought to 0.1% w/v by dilution with 50 mM phosphate buffer (pH 7.0) and the preparation was again ultra-centrifuged for 1.5 h at 105,000 ×*g*. The supernatant (containing the RC) was collected and kept at 4 °C, and the pellet was resuspended at a final OD_{880} of 25 in phosphate buffer and n-dodecyl-β-D-maltoside (DDM) detergent (2% w/v) for 1 h at 4 °C. The DDM concentration was brought to 0.2% w/v by dilution with phosphate buffer and the suspension was ultra-centrifuged for 1.5 h at 105,000 ×*g*. The supernatant, collected at this point, contained the LH1 complex. The OD_{880} of both collected supernatants, *i.e.* the one containing the RC and the other one with the LH1, was measured and adjusted to 2.0 for further analysis.

2.3. Extraction and determination of pigment content

Pigments were extracted from the preparations containing the RC and LH1 complexes isolated from *R. rubrum*. The pigment extraction and determination were performed using established protocols by Küpper et al. [24] with modifications described in Thomas et al. [25]. Briefly, samples were dried under vacuum at room temperature, using a Speed-Vac concentrator (Savant SPD111V) in phosphate buffer (*i.e.* slightly alkaline and minimising Cu^{2+} solubility). After drying, extraction of pigments was performed in 1 mL of 100% v/v acetone, in which metal salts have minimal solubility [26], to prevent artefactual Mg-substitution by Cu^{2+} during the extraction. Afterwards, to extract the more hydrophilic pigments efficiently, a secondary extraction was done with 1 mL of 100% v/v methanol at 4 °C overnight, in which the hydrophilic pigments dominated. However, compared to the acetone extracts, no new pigments were found in the methanol extracts by HPLC-ICP-sfMS, therefore the results primarily refer to the acetone extracts. Pigment quantification was performed by the Gauss Peak Spectra (GPS) method as described by Küpper et al. [27].

2.4. UV/VIS spectroscopy

The spectra of LH1/RC preparations and pigment extracts were measured by using an ultraviolet-visible-near-infrared (UV/VIS/NIR) spectrophotometer (Perkin Elmer Lambda 750).

Whole-cell absorbance spectra of the bacterial cultures were

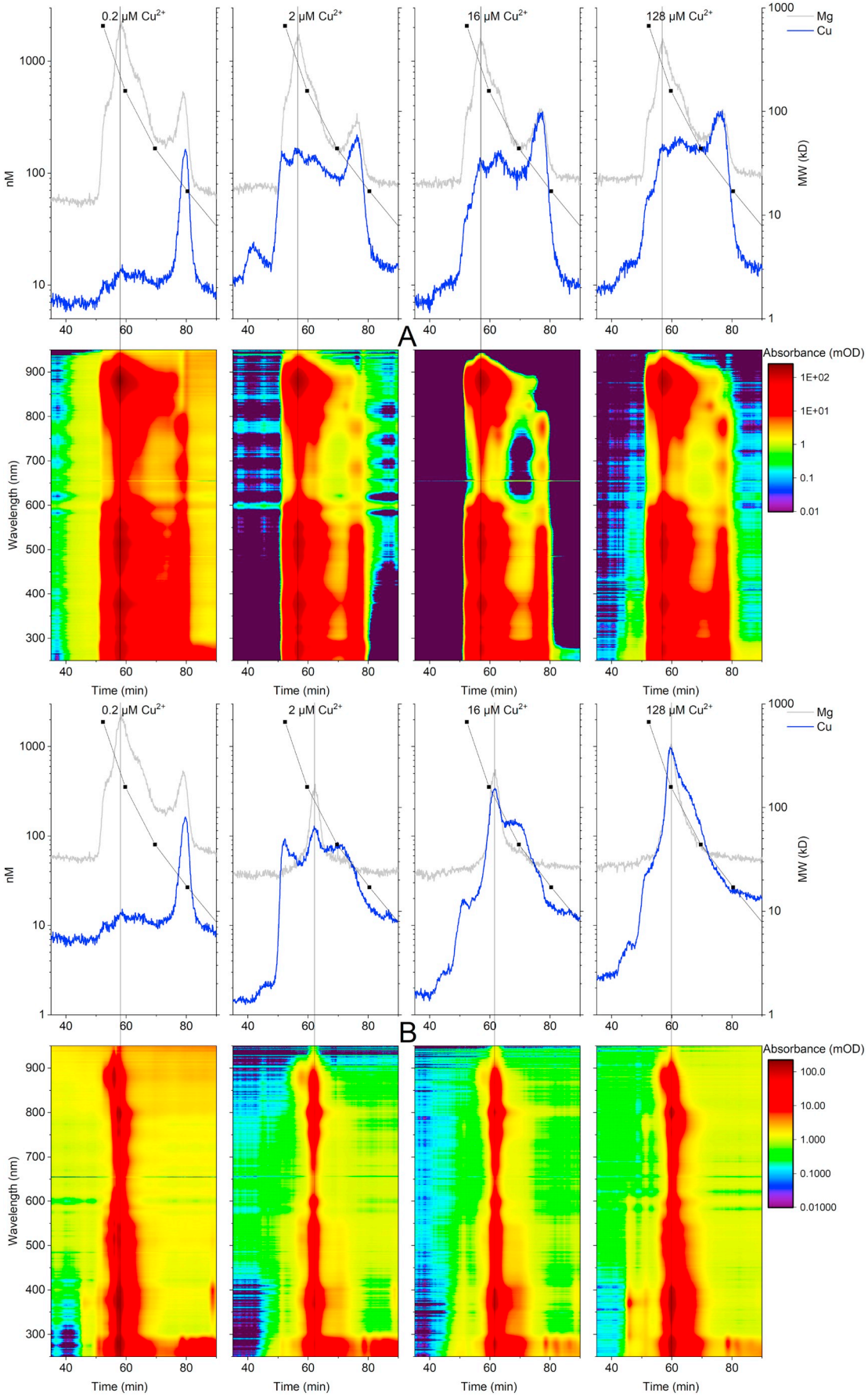

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Fig. 3. HPLC-DAD-ICPsfMS analysis of Cu binding in LH1 preparations from *Rhodospirillum rubrum* cells stressed with different Cu^{2+} concentrations. From left to right, 0.2 (control), 2, 16 and 128 μM of Cu^{2+}. For better clarity of the graphs, the labels for each axis are shown only on the outermost graph. The data are examples from a typical experiment; altogether three experiments were analysed by HPLC-ICPMS of proteins. **A**) LH1; **B**) RC.

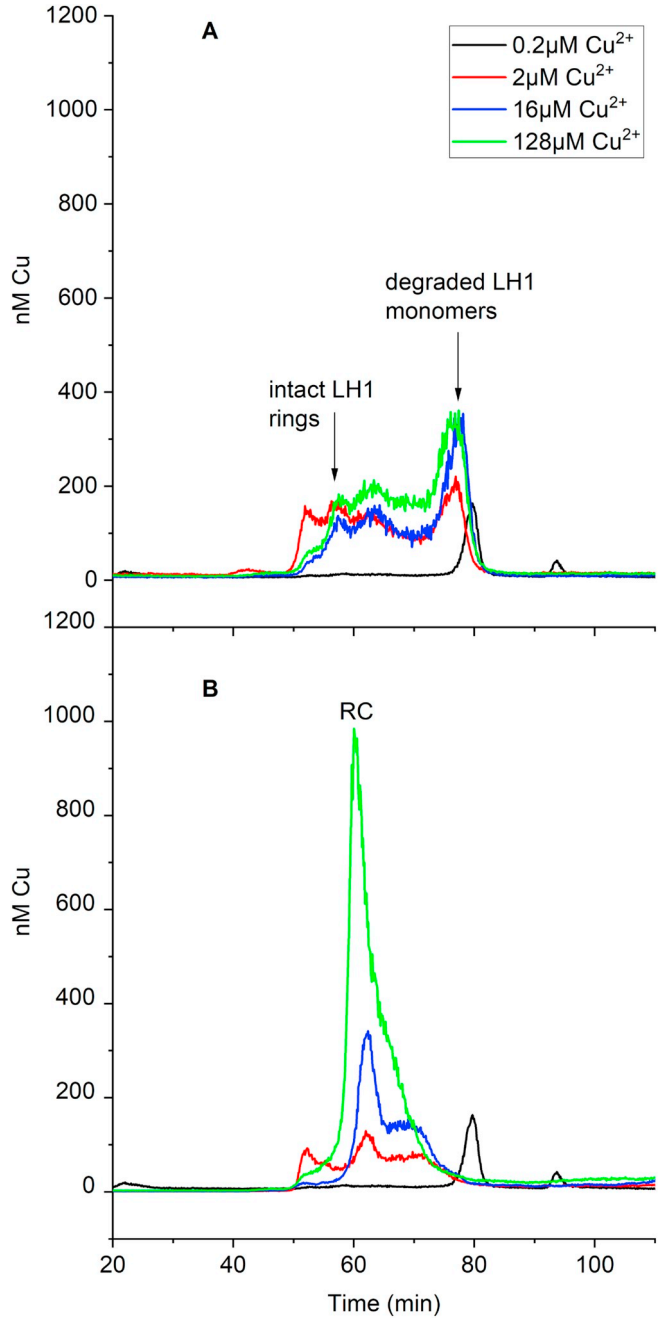

Fig. 4. HPLC-DAD-ICPsfMS analysis of Cu binding in LH1 and RC - comparison of Cu concentrations *via* the ICP-MS measurements; data are from Fig. 2. **A**) LH1; **B**) RC.

recorded with Shimadzu UV-2600 spectrophotometer (Shimadzu, Japan) equipped with an integration sphere.

2.5. In vivo measurements

By measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be obtained [28]. During the flash fluorescence induction (FFI) [29], cells were exposed to a 140 μs long pulse of cyan light (505 nm) while the fluorescence signal (λ > 850 nm) was registered with 10 MHz frequency. The kinetics of fluorescence decay starting 10 μs after the FFI down to 83 s was probed by logarithmically spaced light pulses (800 ns duration) also provided by cyan Luxeon Rebel diodes. For the fluorescence induction, the curves were fitted using Lavergne and Trissl's antenna model [30] neglecting the existence of beta RCs. For the relaxation kinetics, A1, k1, A2, k2, A3, k3 were fitted to a sum of three exponential decays with an offset of F_0. During the measurement, only the sample number 1 was diluted by the medium-200 μL of cells together with 2.2 mL of medium, all the rest was measured as such.

Time-resolved absorption spectroscopy was performed using the locally-build microsecond flash-photolysis instrument [31] using two broadband Xe-pulses (~mJ per pulse) as pump source. Yield of charge separation (formation of oxidized primary donor) was quantified by the amplitude of the electrochromic shift of the accessory BChl a, ΔA_{792}-ΔA_{811} [32]. For the measurement, the bacteria were harvested by centrifugation at 5000 × *g* (10 min) and resuspended in the growth medium. All samples were adjusted to the same OD at 880 nm (~0.3). Measurements were done in 1 × 1 cm fluorescence cuvettes under ambient atmosphere with constant stirring. Cells were dark-adapted for 5 min before start of measurement.

2.6. HPLC-ICP-sfMS measurements

Metal binding to the pigment-protein complexes and the pigments was analysed by a new HPLC-ICPMS system based on a metal-free HPLC system and a sector-field ICP-MS (ICP-sfMS) with desolvating injection, as described in detail in a publication about that system (Küpper et al. [33], submitted). Briefly, proteins were separated by size exclusion chromatography on three 10 × 300 mm metal-free SEC columns in series using an aqueous buffer system, while pigments were separated on a C-18 column with a methanol-acetone gradient. Detection was done by the ICP-sfMS and a diode array detector coupled online to the HPLC. Calibration of the metal concentration measurements in the proteins was done by running metal-EDTA complexes in the same buffer at the same flow rate as the protein extracts, but *via* a column bypass not to contaminate the column. Calibration of the metal concentration measurements in the pigment extracts was done by running standards of Mg-Chl a, Cu-Chl a and Cu-BChl a under the same conditions as the extracts on the column, and then comparing the UV/VIS absorption *via* the molar absorptivity with the metal signal in the ICP-MS. The ICP-sfMS instrument was optimally tuned to reduce the potential interferences by choosing medium resolutions with an acceptably low oxide formation rate as monitored by CeO+/Ce+.

3. Results

3.1. Growth of Rhodospirillum rubrum

The effect of copper on growth of *R. rubrum* was measured by taking the fresh weight of the collected cells after four days of treatment with the different copper concentrations (0.2 μM as control, 2, 16 and 128 μM of $CuSO_4$ as stress). OD_{650} was measured as well (Fig. 1). Already 2 μM Cu^{2+} led to a drastic decrease of FW and OD_{650}, which showed the high sensitivity of these bacteria towards copper toxicity.

3.2. UV/VIS/NIR absorption spectra of LH1 and RC

The pigmentation of the cells and of LH1 remained little affected by Cu^{2+}, showing that the inhibition of growth was not caused by an inhibition of pigment synthesis. In the case of the UV/VIS/NIR absorption

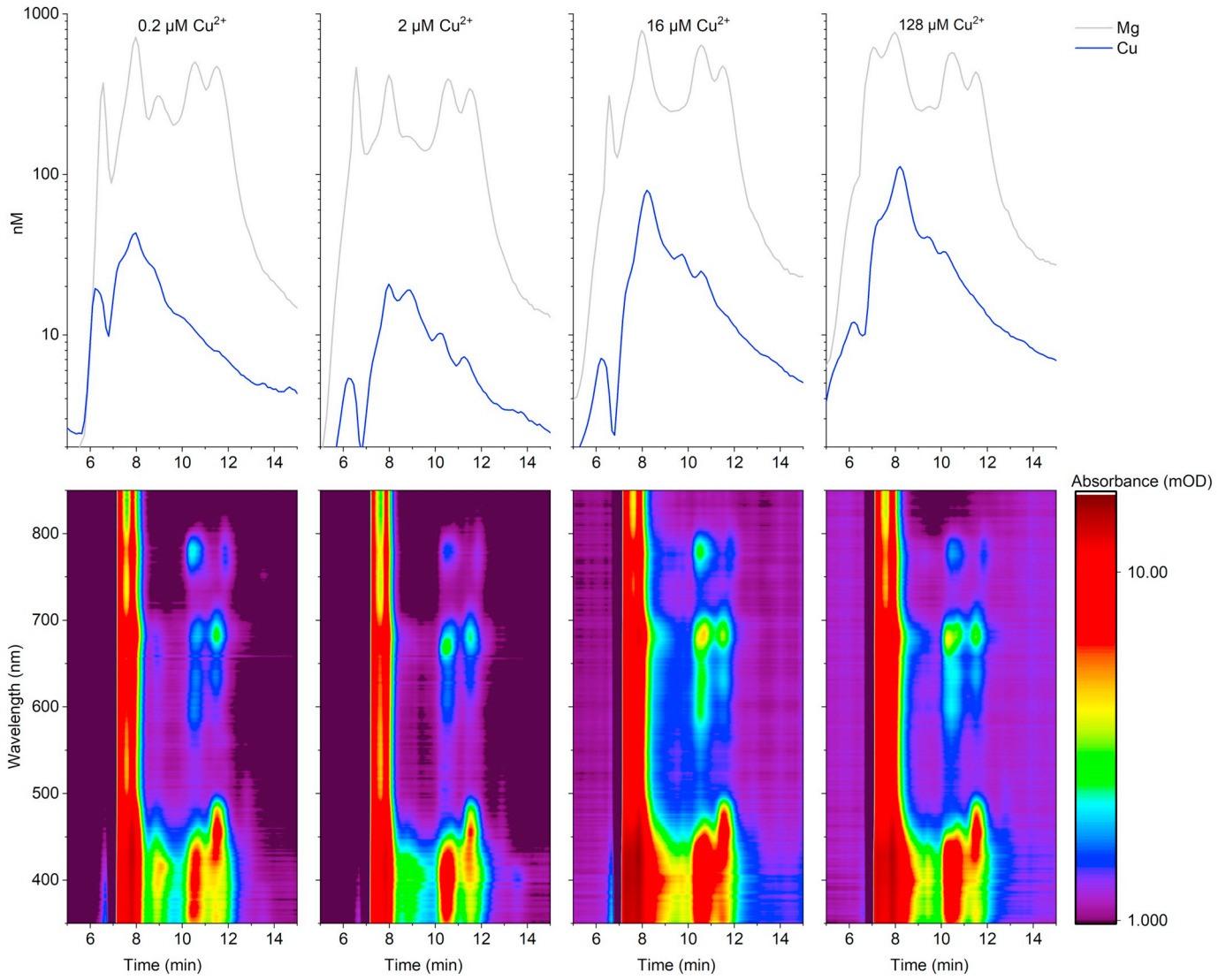

Fig. 5. HPLC-DAD-ICPsfMS analysis of copper binding to pigments of LH1, extracted with methanol from LH1 protein preparations isolated from Cu^{2+}-stressed *Rhodospirillum rubrum* cells. From left to right, 0.2 (control), 2, 16 and 128 µM of Cu^{2+}. Zoomed view of the region with the Cu-binding pigment; see Supplement 2A for the whole chromatogram.

spectra of the isolated RCs, changes in the ratios between individual pigment peaks could be observed (Fig. 2). Further, the shoulder arising at around 600 nm could be indicative of a change in coordination state of the central metal. In order to be able to quantify these effects, HPLC of pigment extracts from the LH1 and RC preparations was performed (see below).

3.3. Binding of copper to LH1 and RC

In order to see in how far the toxicity of Cu^{2+} was correlated to binding of copper to pigment-protein complexes, HPLC-DAD-ICP-sfMS analyses were performed on the isolated LH1 and RC complexes (Figs. 3, 4, 5). LH1 eluted at about 58 min and RC at about 62 min (Fig. 3). In LH1 of cells grown on 0.2 µM Cu^{2+} ("control") there was no significant amount of Cu binding. But already in the LH1 of cells grown on 2 µM Cu^{2+}, there was clearly Cu^{2+} binding in LH1 (Fig. 4A). This increased only little at 16 µM and 128 µM Cu^{2+}, likely meaning that only few of the BChls are easily accessible to Cu^{2+}. In addition to the Cu-peak correlated to the intact LH1, in the cells stressed with Cu^{2+} a small peak of LH1 monomers (judged by the size) appeared at about 76 min, which correlated with an even higher Cu peak in the chromatogram than for the intact LH1 (Figs. 3A, 4). Again, Cu-binding saturated; it increased only little the 16 µM to 128 µM Cu^{2+} treatment. The protein eluting at this time displayed a UV/VIS absorption spectrum indicating degraded BChl (Supplement 1).

The measurements on the RC showed that it very strongly, much more than the LH1, binds Cu^{2+} (Fig. 4). Further, there was still a strong increase in Cu^{2+} binding to the RC from the 2 µM Cu^{2+} treatment to the 16 µM Cu^{2+} and 128 µM Cu^{2+} treatments (Fig. 4B). At 2 µM Cu^{2+}, the ratio of Cu to Mg in the reaction centre was already about 1:3.1, which increased to 1:1.6 in the 16 µM Cu^{2+} treatment and almost 1:1 in the 128 µM Cu^{2+} treatment (Fig. 3B).

3.4. Binding of copper to pigments extracted from the pigment-protein complexes

As shown already by the data on the protein level, Cu^{2+} bound much more in the RCs than in LH1, but on the pigment level the difference was still much more drastic. In acetone extracts of LH1, only traces of Cu-binding pigments could be found, and no clearly Cu-related pigment peak was observed (Supplement 2A). In the methanol extracts aimed at recovering highly hydrophilic pigments that are not efficiently extracted with acetone, a Cu-binding pigment eluted at about 8 min and this peak increased with the increasing copper treatments (Fig. 5,

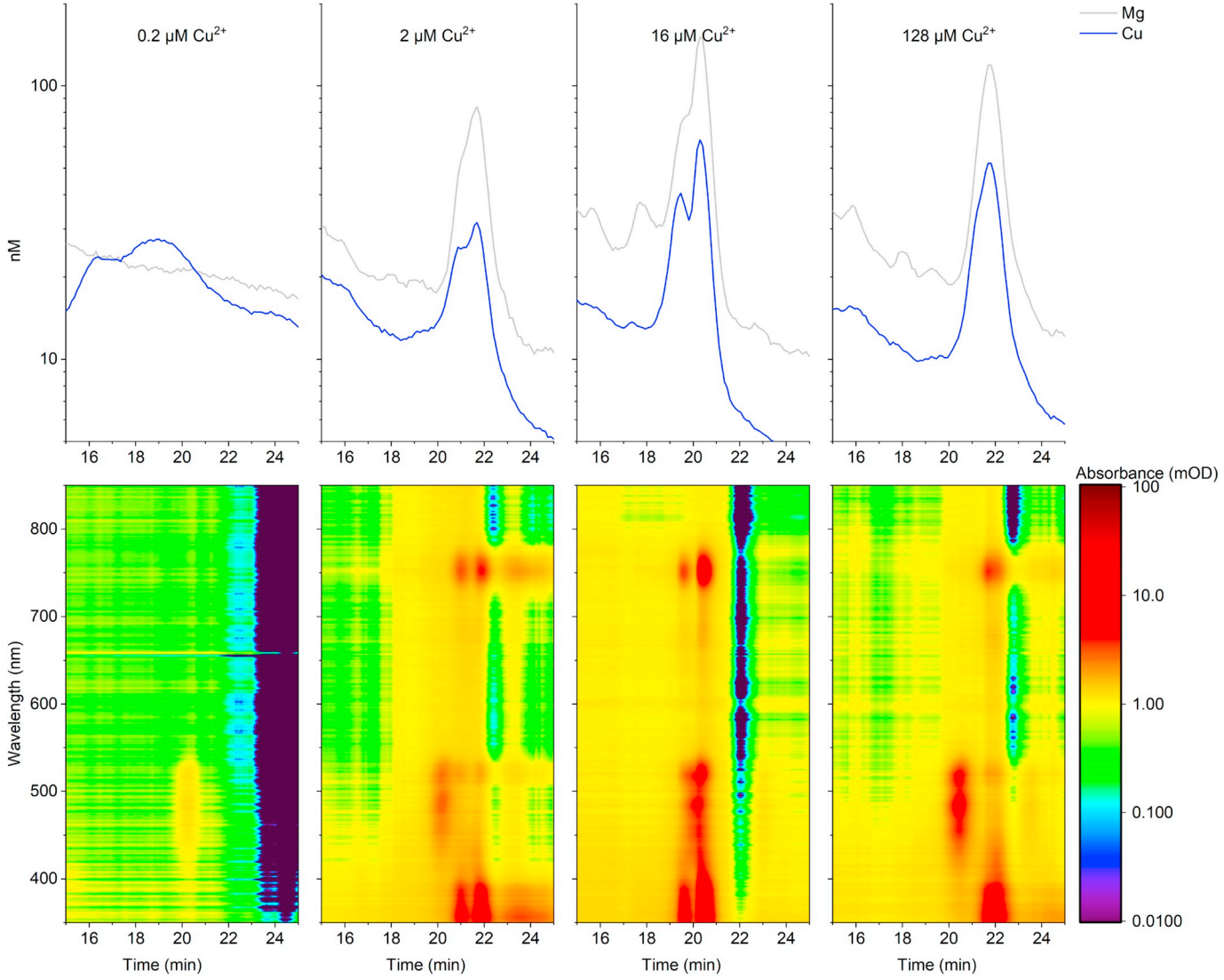

Fig. 6. HPLC-DAD-ICPsfMS analysis of Cu binding in RC, extracted with acetone from RC protein preparations isolated from Cu-stressed *Rhodospirillum rubrum* cells. From left to right, 0.2 (control), 2, 16 and 128 µM of Cu^{2+}. Zoomed view of the region with the Cu-binding pigment; see Supplement 3 for the whole chromatogram. The data are examples from a typical experiment; altogether 3 exp. were analysed by HPLC-ICPMS of pigments.

Supplement 1C). However, it was not well resolved and less abundant than an Mg-binding BChl degradation product eluting almost at the same time, so that an identification was not possible.

In contrast, in the RC extracts, the rise of a Cu peak and absorption signal with characteristics of a porphyrin spectrum could be found at about 20 min elution time (Supplement 3, Fig. 6). A closer view showed that in the extracts from the 2 and 16 µM Cu^{2+} treatments it actually consisted of two peaks, at 19.5 and 20.5 min, which merged to one at 21.9 min in the extract from the 128 µM Cu^{2+} treatment (Fig. 6, Supplement 1D). The Cu-correlated pigment peaks were not native [Cu]-BChl, as shown by comparison of their spectra (Supplement 1D) with literature data [34] and by running a Cu-BChl standard under the same conditions as the RC-extracts (Supplement 4). Nevertheless, they were clearly spectra of porphyrins with all their characteristic features, and the direct correlation (timing and intensity) of the ICP-MS signal with the signal from the DAD clearly showed that they are copper complexes although the spectrum looks more like BPhe [34]. Thus, they must be degradation products of [Cu]-BChl, as BChl precursors would not have been in the pigment-protein complexes. On the basis of the concentration ratios observed in the HPLC-ICPMS chromatograms of the pigments, the Cu was in the range of some percent of total metal bound to porphyrins, but an accurate quantification was not possible due to the multiple degradation products formed, with often not well quantifiable peaks (Supplement 2). In methanol extracts of the RC preparations, no new Cu-binding pigments were found in comparison to the acetone extracts (not shown).

3.5. *In vivo measurements*

Effect of copper on the photosynthetic apparatus of *R. rubrum* was apparent at the whole cell level, as shown in Fig. 7 that presents absorbance spectra of the bacterial cultures in the near-infrared region. As seen there, in all the copper-treated cultures, the absorption band at 800 nm corresponding to the accessory BChl a of the RC and to the upper excitonic band of the special pair was shifted to higher energies compared to the control (0.2 µM Cu^{2+}) culture. Electrochromic blue shift of the absorption of the accessory BChl a is known to occur following the oxidation of the primary donor in the purple-bacterial RC [35]. This would indicate that the copper-treated cells contained a pool of oxidized RC. To test this possibility, the 128 µM culture was treated with the reductant sodium dithionite (~1 mM final concentration). As seen in the inset of Fig. 7, this treatment led to a shift of the band to the lower energies, to a position matching its spectrum in the control sample. This supported the interpretation of the absorbance difference

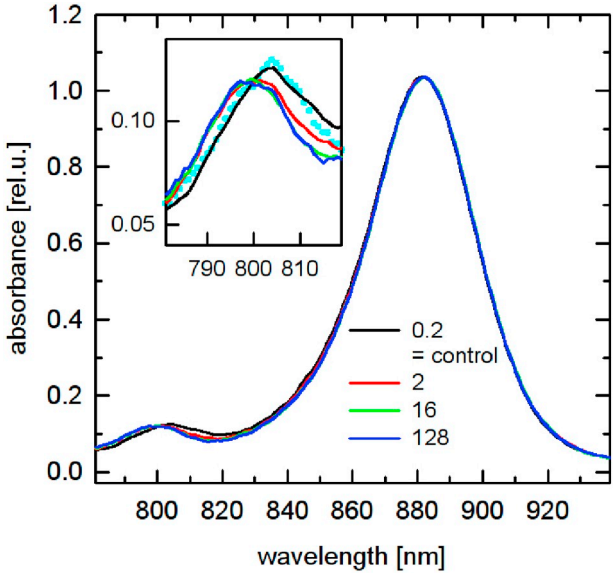

Fig. 7. Near-infrared region of absorbance spectrum of *R. rubrum* cultures grown at different Cu^{2+} concentrations (given in μM in the legend). Spectra were measured using an integration sphere and normalized at 881 nm. Inset shows expanded region corresponding to the absorption band of the accessory BChl a in the RC. The cyan symbols in the inset show the absorption of the culture grown at 128 μM Cu^{2+} (blue line) copper after treatment by the reductant sodium dithionite.

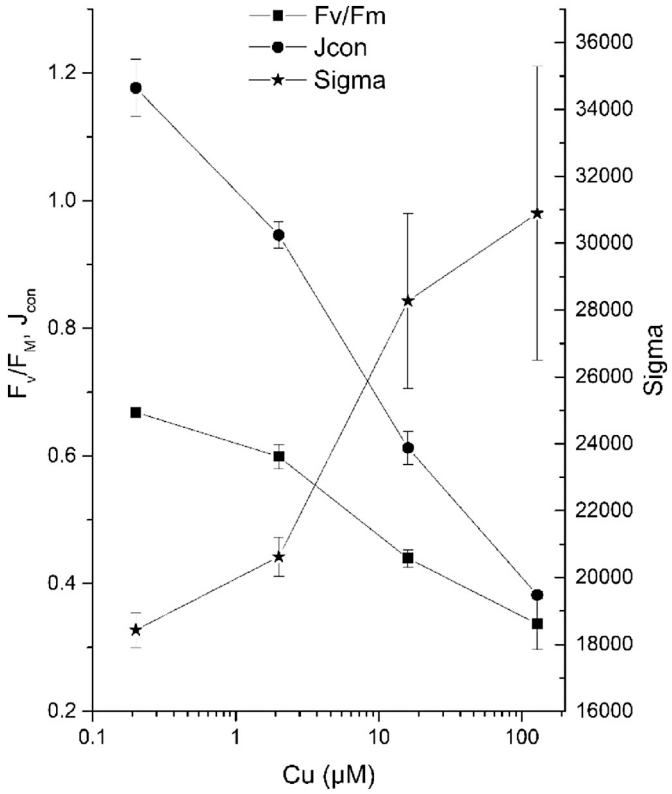

Fig. 8. Parameters calculated from the fast fluorescence rise in living cells. The left ordinate shows maximal dark-adapted quotochemical quantum yield (F_v/F_M) and connectivity (J_{con}), the right ordinate shows antenna cross-section (Sigma). Data are averages ± SE of three experiments.

between control and copper-treated cells being due to presence of oxidized RC in the latter.

Changes in photosynthetic light reactions, and thus the relevance of the copper binding to the pigment-protein complexes and pigments, were identified using fluorescence kinetic analysis. Both the rise from the minimal fluorescence quantum yield in dark-adapted state (F_0) to the maximal fluorescence quantum yield during a supersaturating flash (F_M) and the relaxation kinetics from F_M to the dark-adapted state, reflecting the re-oxidation of Q_A, were analysed.

The parameter F_V/F_M, which measures the maximum quantum efficiency of RC photochemistry in dark adapted state, decreased at higher Cu^{2+} concentrations (Fig. 8). This was accompanied by a decrease of the photosystem connectivity, but an increase of the relative antenna size per RC, all calculated from the same measurements of fast fluorescence induction (Fig. 8). All treatments were significantly different ($P \leq 0.05$).

The raw data of the reoxidation kinetics at increasing copper concentrations show that the copper-stressed cells generally had less fluorescence (Fig. 9A). Rate constants of the fluorescence decay revealed that the control (0.2 μM Cu^{2+}) can be fitted with one fast component (3500 s^{-1}) and two additional slow components (16 s^{-1}, 0.05 s^{-1}). At toxic Cu^{2+} concentrations, the fast component (Q_A^- to Q_B electron transfer) became retarded more drastically, but the others became slower as well (Fig. 9B).

Flash-photolysis data, measuring the photooxidation of the primary donor, showed a strong decrease already at 2 μM Cu^{2+}, and almost complete quenching of activity at 16 and 128 μM Cu^{2+} (Fig. 10). This could be related to the already oxidized state of the RC as measured by the absorption spectra (Fig. 7).

4. Discussion

This work has shown that copper toxicity to *R. rubrum* occurs already at low, environmentally relevant concentrations, and is related to a specific, strong inhibition of the photosynthetic light reactions. This finding is important because only very few earlier studies dealt with copper toxicity in phototrophic bacteria. In those, either much higher copper concentrations were applied, which led to entirely different effects, or inhibition of photosynthesis was not investigated.

Liotenberg et al. [19] reported, looking at *Rubrivivax gelatinosus*, that BChl synthesis was inhibited completely. However, in that study 1.6 μM Cu was regarded as the control, further copper was added for only 24 h and in high concentrations (50–200 μM), and the strong inhibition of BChl biosynthesis occurred only under oxic conditions, while in the current study anoxic conditions were applied.

The current results have shown that Cu binds in a high-affinity and saturable way to LH1 and the RC at sublethal Cu concentrations, correlating to the growth of the cells. When observing the absorbance spectra at pigment level, we can be sure that at least some of the Cu that was already observed at the protein level was actually present in the bacteriochlorophyll.

From the HPLC-ICPsfMS analysis of acetone extracts from RC preparations it is concluded that degradation products from [Cu]-BChl were formed. When these Cu-complexes occurred, Mg-complexes with similar features (*i.e.* [Mg]-BChl degradation products) occurred as well. Their elution times from the HPLC column unfortunately overlapped with the Cu-complexes, so that the measured spectra do not represent pure substances. But as there were no similar degradation products of [Mg]-BChl detectable in the control, and at room conditions the Cu should stay stably in its divalent form, it is most likely that the degradation of the [Cu]-BChl occurred already in the stressed cells, and the damage to the cells/proteins led to degradation of some [Mg]-BChl along with the [Cu]-BChl. For LH1, the formation of [Cu]-BChl may have contributed to the dissociation of trimers during Cu^{2+} toxicity, as the Cu-complexes of (B)Chl lack the axial ligands [10] that are required for binding the pigment in light harvesting complexes [11] and for stabilising the protein structure of these complexes [12].

Still, one group of authors suggested binding of Cu to the RC protein, based on short-term *in vitro* binding assays with very high Cu

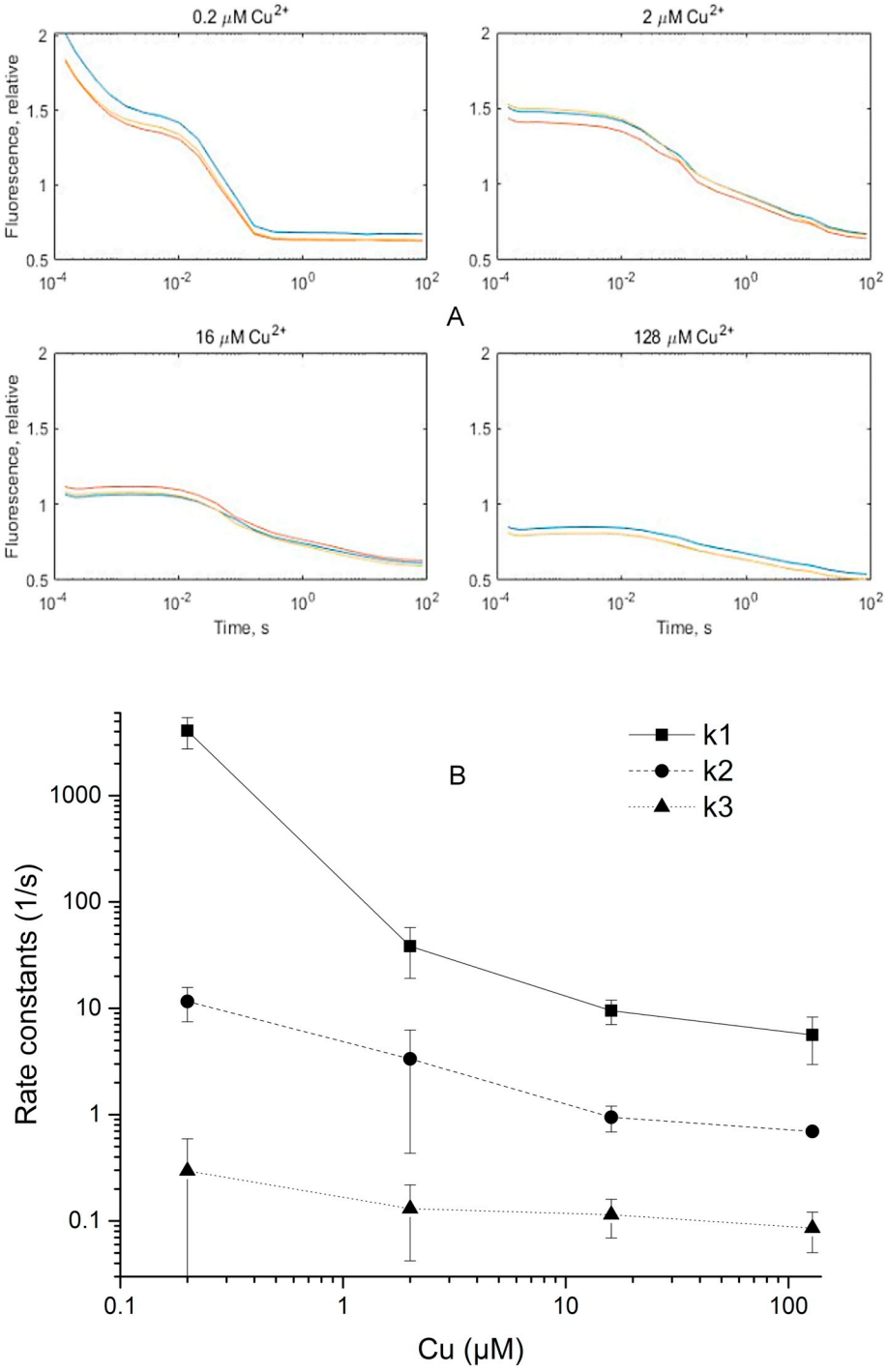

Fig. 9. Chlorophyll fluorescence decay after F_M measured to determine the re-oxidation of Q_A. **A**) Examples of chlorophyll fluorescence relaxation curves used for the calculation of the rate constants; **B**) Rate constants calculated from these curves. Data are averages ± SE of three experiments.

concentrations (10× molar excess) [36,37]. The conclusion of the binding site, however, is based on ESR data that primarily indicate nitrogen atoms in close proximity of the copper. The authors of that study interpreted these nitrogen atoms as originating from histidines, but the current study suggests that they might originate from the nitrogen ligands inside the porphyrin ring of BChl and its degradation products. While a lot of research has been done on degradation pathways of chlorophylls that involve a transmetallation, only little is known about that for bacteriochlorophylls. One article reported that Cu bound in homologues of BChl e in sediments of a lake [38], but in fact BChl e is not a bacteriochlorin, it is a chlorin like Chl.

All the results of the *in vivo* experiments of the current study primarily show a very strong inhibition of the RC. The parameter F_V/F_M, which measures the maximum quantum efficiency of RC photochemistry in dark adapted state, decreased towards the higher Cu concentrations. This is consistent with the data found by Küpper et al. [7] in algae and higher plants, in which Cu binding to the PSII RC during the *sun reaction* caused a strong decrease of F_V/F_M. Based on *in vivo* and pigment extract data, at that time the conclusion was that the inhibition was caused by Cu binding to the Phe a of the RC [7]. The flash photolysis data of the current study strongly indicate an inhibition of primary charge separation or a permanent charge separation that

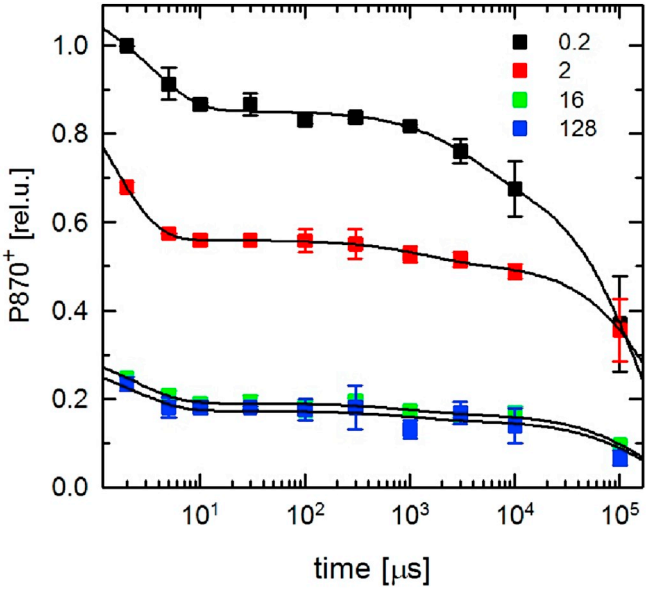

Fig. 10. Analysis of RC activity by subjecting the RC preparation to a 2 μs actinic flash. Kinetic data showing the decay of the photooxidized primary donor.

persists even in the dark. Both is consistent with the data from HPLC-ICPsfMS as [Cu]-BChl or its degradation products would not be suitable for proper function of the RC. The data from the fast fluorescence rise suggest an increase in antenna cross-section, decrease of antenna connectivity, and an inhibition of the electron transfer from Q_A to Q_B. It is possible that these are independent effects, but they might also be linked to each other. In particular, while it is difficult to imagine existence of a mechanism responsible for the increase in LH1 optical cross section within on LH1-RC assembly, the transfer of excitons among the photosynthetic units within the chromatophores is fast and efficient. Therefore, the increase in antenna cross-section could be explained by a mixture of LH1-RC assemblies with defunct RCs containing Cu, and assemblies with intact RCs containing Mg. Excitons from the defunct assemblies would be transferred to the intact assemblies, which would result in lower J and elevated sigma in the induction curve fits of the ensemble population of RCs, even though all LH1 complexes retain the same size. This is also in agreement with the observation of a pool of oxidized RC, since the rate constant of the excitation trapping by oxidized primary donor is $\sim(200\ \mathrm{ps})^{-1}$, about 4× slower than trapping by the open RC [39]. At the current state of analysis, this seems to be the most logical explanation on the basis of all results. Further studies will aim at resolving the mechanism in more detail.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2019.06.004.

Declaration of Competing Interest

The authors declare that they have no conflict of interest in any of the points stated in the journal guidelines cited below, and have reported all required information:

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References

[1] H. Küpper, E. Andresen, Mechanisms of metal toxicity in plants, Metallomics. 8 (2016) 269–285, https://doi.org/10.1039/c5mt00244c.
[2] E. Andresen, E. Peiter, H. Küpper, Trace metal metabolism in plants, J. Exp. Bot. 69 (2018) 909–954, https://doi.org/10.1093/jxb/erx465.
[3] H. Küpper, F.C. Küpper, M. Spiller, [Heavy metal]-chlorophylls formed *in vivo* during heavy metal stress and degradation products formed during digestion, extraction and storage of plant material, in: B. Grimm, R.J. Porra, W. Rüdiger, H. Scheer (Eds.), Chlorophyll Bacteriochlorophylls Biochem. Biophys. Funct. Appl, Springer, Dordrecht, 2006, pp. 67–77, , https://doi.org/10.1007/1-4020-4516-6_5.
[4] T. Watanabe, M. Kobayashi, Chlorophylls as functional molecules in photosynthesis - molecular composition *in vivo* and physical chemistry *in vitro*, Spec. Artic. Coord. Chem. Biol. Important Subst. (1988) 383–395, https://doi.org/10.1246/nikkashi.1988.383.
[5] K. Karukstis, Chlorophyll fluorescence as a physiological probe of the photosynthetic apparatus, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, Ann Arbor, Boston, London, 1991, pp. 769–795.
[6] H. Küpper, F. Küpper, M. Spiller, *In situ* detection of heavy metal substituted chlorophylls in water plants, Photosynth. Res. 58 (1998) 123–133, https://doi.org/10.1023/A:1006132608181.
[7] H. Küpper, I. Šetlík, M. Spiller, F.C. Küpper, O. Prášil, Heavy metal-induced inhibition of photosynthesis: targets of *in vivo* heavy metal chlorophyll formation, J. Phycol. 38 (2002) 429–441, https://doi.org/10.1046/j.1529-8817.2002.t01-1-01148.x.
[8] L. Fiedor, D. Leupold, K. Teuchner, B. Voigt, C.N. Hunter, A. Scherz, H. Scheer, Excitation trap approach to analyze size and pigment - pigment coupling: reconstitution of LH1 antenna of *Rhodobacter sphaeroides* with Ni-substituted bacteriochlorophyll, Biochemistry. 40 (2001) 3737–3747, https://doi.org/10.1021/bi002257f.
[9] T. Watanabe, K. Machida, H. Suzuki, M. Kobayashi, K. Honda, Photoelectrochemistry of metallochlorophylls, Coord. Chem. Rev. 64 (1985) 207–224, https://doi.org/10.1016/0010-8545(85)80051-5.
[10] L.J. Boucher, J.J. Katz, Aggregation of metallochlorophylls, J. Am. Chem. Soc. 89 (1967) 4703–4708, https://doi.org/10.1021/ja00994a024.
[11] C. Rebeiz, F. Belanger, Chloroplast biogenesis 46. Calculation of net spectral shifts induced by axial ligand coordination in metalated tetrapyrroles, Spectrochim. Acta 40A (1984) 793–806, https://doi.org/10.1016/0584-8539(84)80170-1.
[12] H. Paulsen, B. Finkenzeller, N. Kühlein, Pigments induce folding of light-harvesting chlorophyll a/b-binding protein, Eur. J. Biochem. 215 (1993) 809–816, https://doi.org/10.1111/j.1432-1033.1993.tb18096.x.
[13] N. Wakao, N. Yokoi, N. Isoyama, A. Hiraishi, K. Shimada, M. Kobayashi, H. Kise, M. Iwaki, S. Itoh, S. Takaichi, Y. Sakurai, Discovery of natural photosynthesis using Zn-containing bacteriochlorophyll in an aerobic bacterium *Acidiphilium rubrum*, Plant Cell Physiol. 37 (1996) 889–893, https://doi.org/10.1093/oxfordjournals.pcp.a029029.
[14] W.S. Kim, Copper replacement of magnesium in the chlorophylls and bacteriochlorophyll, Z. Naturforsch. 22 (1967) 1054–1061, https://doi.org/10.1515/znb-1967-1014.
[15] M. Yamamura, M. Kobayashi, K. Inoue, M. Hara, N. Wakao, H. Kano, T. Watanabe, M. Akiyama, H. Kise, *Acidiphilium Rubrum* and zinc-bacteriochlorophyll part 3: high resistance of zinc-bacteriochlorophyll a to acid, in: G. Garab (Ed.), Photosynth. Mech. Eff, Springer, Dordrecht, 1998, pp. 739–742, , https://doi.org/10.1007/978-94-011-3953-3_173.
[16] E. Esmarch, Über die Reinkultur eines Spirillum., Zentralblatt Für Bakteriol. Parasitenkunde, Infekt. Und Hyg. Abteilung, 1887, pp. 225–230.
[17] H. Molisch, Die purpurbakterien: nach neuen untersuchungen, *Jena I–VII*, https://www.zobodat.at/pdf/MON-ALLGEMEIN_0207_0001-0095.pdf, (1907).
[18] A.C. Munk, A. Copeland, S. Lucas, A. Lapidus, T.G. Del Rio, K. Barry, J.C. Detter, N. Hammon, S. Israni, S. Pitluck, T. Brettin, D. Bruce, C. Han, R. Tapia, P. Gilna,

J. Schmutz, F. Larimer, M. Land, N.C. Kyrpides, K. Mavromatis, P. Richardson, M. Rohde, M. Göker, H.-P. Klenk, Y. Zhang, G.P. Roberts, S. Reslewic, D.C. Schwartz, Complete genome sequence of *Rhodospirillum rubrum* type strain (S1T), Stand. Genomic Sci. 4 (2011) 293–302, https://doi.org/10.4056/sigs.1804360.

[19] S. Liotenberg, A.-S. Steunou, A. Durand, M.-L. Bourbon, D. Bollivar, M. Hansson, C. Astier, S. Ouchane, Oxygen-dependent copper toxicity: targets in the chlorophyll biosynthesis pathway identified in the copper efflux ATPase CopA deficient mutant, Environ. Microbiol. 17 (2015) 1963–1976, https://doi.org/10.1111/1462-2920.12733.

[20] S. Panwichian, D. Kantachote, B. Wittayaweerasak, M. Mallavarapu, Isolation of purple nonsulfur bacteria for the removal of heavy metals and sodium from contaminated shrimp ponds, Electron. J. Biotechnol. 13 (2010), https://doi.org/10.2225/vol13-issue4-fulltext-8 (0–0).

[21] L.J. Bird, M.L. Coleman, D.K. Newman, Iron and copper act synergistically to delay anaerobic growth of bacteria, Appl. Environ. Microbiol. 79 (2013) 3619–3627, https://doi.org/10.1128/aem.03944-12.

[22] G. Cohen-Bazire, W.R. Sistrom, R.Y. Stanier, Kinetic studies of pigment synthesis by non-sulfur purple bacteria, J. Cell. Comp. Physiol. Banner. 49 (1957) 25–68 https://onlinelibrary.wiley.com/doi/abs/10.1002/jcp.103 0490104.

[23] R. Picorel, G. Bélanger, G. Gingras, Antenna holochrome B880 of *Rhodospirillum rubrum* S1. Pigment, phospholipid, and polypeptide composition, Biochemistry. 22 (1983) 2491–2497, https://doi.org/10.1021/bi00279a028.

[24] H. Küpper, A. Parameswaran, B. Leitenmaier, M. Trtílek, I. Šetlík, Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*, New Phytol. 175 (2007) 655–674, https://doi.org/10.1111/j.1469-8137.2007.02139.x.

[25] G. Thomas, H.J. Stärk, G. Wellenreuther, B.C. Dickinson, H. Küpper, Effects of nanomolar copper on water plants - comparison of biochemical and biophysical mechanisms of deficiency and sublethal toxicity under environmentally relevant conditions, Aquat. Toxicol. 140–141 (2013) 27–36, https://doi.org/10.1016/j.aquatox.2013.05.008.

[26] M. Li, D. Constantinescu, L. Wang, A. Mohs, J. Gmehling, Solubilities of NaCl, KCl, LiCl, and LiBr in methanol, ethanol, acetone, and mixed solvents and correlation using the LIQUAC model, Ind. Eng. Chem. Res. 49 (2010) 4981–4988, https://doi.org/10.1021/ie100027c.

[27] H. Küpper, S. Seibert, A. Parameswaran, Fast, sensitive, and inexpensive alternative to analytical pigment HPLC: quantification of chlorophylls and carotenoids in crude extracts by fitting with Gauss peak spectra, Anal. Chem. 79 (2007) 7611–7627, https://doi.org/10.1021/ac070236m.

[28] K. Maxwell, G.N. Johnson, Chlorophyll Fluorescence - A Practical Guide, 51 (2000), pp. 659–668, https://doi.org/10.1093/jxb/51.345.659.

[29] L. Nedbal, M. Trtílek, D. Kaftan, Flash fluorescence induction: a novel method to study regulation of photosystem II, J. Photochem. Photobiol. B Biol. 48 (1999) 154–157, https://doi.org/10.1016/S1011-1344(99)00032-9.

[30] J. Lavergne, H.W. Trissl, Theory of fluorescence induction in photosystem II: derivation of analytical expressions in a model including exciton-radical-pair equilibrium and restricted energy transfer between photosynthetic units, Biophys. J. 68 (1995) 2474–2492, https://doi.org/10.1016/S0006-3495(95)80429-7.

[31] D. Bína, R. Litvín, F. Vácha, P. Šiffel, New multichannel kinetic spectrophotometer–fluorimeter with pulsed measuring beam for photosynthesis research, Photosynth. Res. 88 (2006) 351–356, https://doi.org/10.1007/s11120-006-9071-y.

[32] D. Bína, R. Litvin, F. Vacha, Kinetics of *in vivo* bacteriochlorophyll fluorescence yield and the state of photosynthetic apparatus of purple bacteria, Photosynth. Res. 99 (2009) 115–125, https://doi.org/10.1007/s11120-009-9408-4.

[33] H. Küpper, S. N. H. Bokhari, N. Jaime-Pérez, L. Lyubenova, N. Ashraf, E. Andresen, Ultra-Trace Metal Speciation Analysis by Coupling of Sector-Field ICP-MS to High-Resolution Size Exclusion and Reversed-Phase Liquid Chromatography, Submitted to Analytical Chemistry. Manuscript ID: ac-2019-002225.

[34] G. Hartwich, L. Fiedor, I. Simonin, E. Cmiel, W. Schäfer, D. Noy, A. Scherz, H. Scheer, Metal-substituted bacteriochlorophylls. 1. Preparation and influence of metal and coordination on spectra, J. Am. Chem. Soc. 120 (1998) 3675–3683, https://doi.org/10.1021/ja970874u.

[35] N.W. Woodbury, J.P. Allen, The pathway, kinetics and thermodynamics of electron transfer in wild type and mutant reaction centers of purple nonsulfur bacteria, Anoxygenic Photosynth. Bact, Kluwer Academic Publishers, Dordrecht, 1995, pp. 527–557, , https://doi.org/10.1007/0-306-47954-0_24.

[36] L.M. Utschig, O. Poluektov, D.M. Tiede, M.C. Thurnauer, EPR investigation of Cu^{2+}-substituted photosynthetic bacterial reaction centers: evidence for histidine ligation at the surface metal site, Biochemistry. 39 (2000) 2961–2969, https://doi.org/10.1021/bi991800t.

[37] L.M. Utschig, O. Poluektov, S.L. Schlesselman, M.C. Thurnauer, D.M. Tiede, Cu^{2+} site in photosynthetic bacterial reaction centers from *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodopseudomonas viridis*, Biochemistry. 40 (2001) 6132–6141, https://doi.org/10.1021/bi0029191.

[38] N. Ohkouchi, Y. Nakajima, H. Okada, H. Kitazato, Copper-chelated bacteriochlorophyll e homologues in sediment from an anoxic lake (Lake Abashiri, Japan), Org. Geochem. 36 (2005) 1576–1580, https://doi.org/10.1016/j.orggeochem.2005.08.003.

[39] R. van Grondelle, Excitation energy transfer, trapping and annihilation in photosynthetic systems, Biochim. Biophys. Acta - Rev. Bioenerg. 811 (1985) 147–195, https://doi.org/10.1016/0304-4173(85)90017-5.