ESRF	**Experiment title:** Cadmium tolerance in the model alga *Chlamydomonas reinhardtii* and mutants	**Experiment number**: EV 58
Beamline: ID21	**Date of experiment**: from: 25/09/2013 to: 01/10/2013	**Date of report**: 28/02/2014
Shifts: 18	**Local contact(s)**: Hiram Castillo-Michel and Giulia Veronesi	*Received at ESRF:*

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Objective and expected results

Chlamydomonas reinhardtii is well-known as an eukaryotic photosynthetic model for the studies of metal stress. It is tolerant to cadmium but the mechanisms involved in this tolerance are still unknown. The synthesis of thiolated peptides (phytochelatins, glutathione...) and the cell-wall could play an important role in its cadmium tolerance. The aim of our experiment was to determine the subcellular compartment of Cd accumulation as well as its chemical forms and to clarify the involvement of chloroplasts and cell wall using specific mutants.

For this experiment, we selected three *C. reinhardtii* strains with mutations related to cadmium tolerance. A cell-wall less (cw 15) strain was used to examine the role of the cell-wall in cadmium uptake, and a (pcs1) mutant, able to synthetize phytochelatin synthase in its chloroplast was studied. Both mutants and wild-type were exposed to 70 µM Cd in order to investigate variations of cadmium localization and speciation related to the toxicity. For that purpose, we used a combination of chemical mapping using X-ray Fluorescence (µXRF) and X-ray Absorption Near Edge Structure spectroscopy (XANES and µXANES) at Cd L_{III}-edge at ID21 beamline.

Results and conclusions of the study

Chlamydomonas reinhardtii (wt, cw 15 and pcs1 strains) was cultivated mixotrophically in 150 ml of Tris-Acetate-Phosphate (TAP) medium at 22°C under constant illumination and agitation (120 rpm). Only for wt strain, we also modified the TAP medium (mTAP) with the intention of increasing the concentration of bioavailable cadmium. A growth decrease was measured in this condition. The $CdCl_2$ (0, 70 µM) was added 24h after medium inoculation. Samples were taken 48 h after cadmium addition, during exponential growth phase. Cells were washed with water to remove adsorbed cadmium on the cell-wall. Two sample preparations were implemented: cells deposits on ultralene membrane to see whole cells and pressed pellet of cells for

bulk analysis. Cadmium, phosphorus and sulphur distributions were determined by µXRF at 3.57 keV in deposits. Cadmium ligands were determined by Cd LIII-edge µXANES in cadmium regions of interest evidenced by µXRF imaging as well as by XANES in bulk samples. The beam size on the sample was 0.6 µm (H) x 0.2 µm (V). All measurements were performed at -180°C using a cryostat to avoid metal redistribution and speciation change.
µXRF data were treated using PYMCA software and µXANES spectra using Athena software. The µXANES spectra were then compared to spectra of Cd model-compounds and fitted by linear combinations of these reference spectra.

For the wild type strain, in classic TAP medium, cadmium was co-located with phosphorus in sub-micrometer sized spots (Figure 1), in contrast with sulfur, which was present in the whole cell and in a bigger spot. The size of the sulfur spot might correspond to the nucleus or to the pyrenoïd. Concerning the speciation, bulk analysis showed that cadmium was predominantly complexed by sulfur ligands (Figure 5), thus suggesting the importance of thiolated compounds. µXANES on the cadmium rich-spots showed that cadmium was predominantly complexed by oxygen ligands (Spot 1, Figure 5). These complementary results could mean that thiolated peptides synthesis was the main but not the only mechanism against cadmium stress in *C. reinhardtii*.

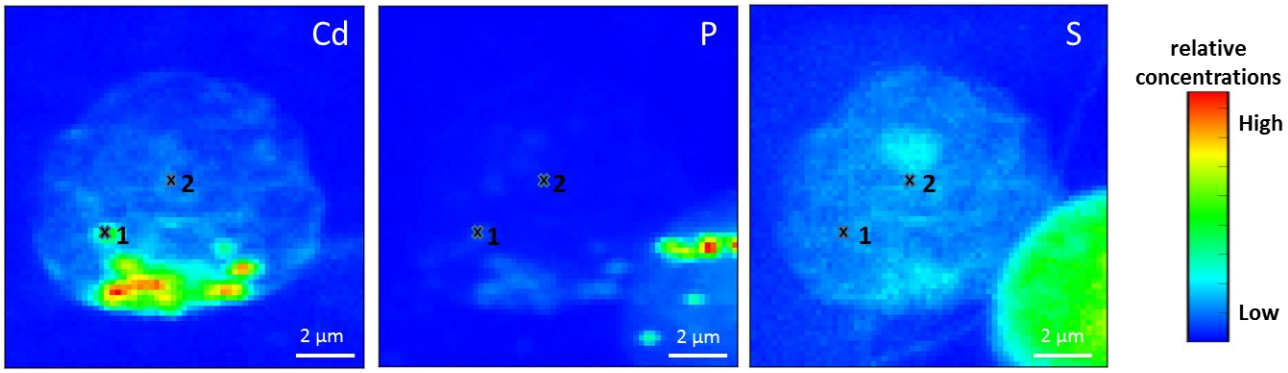

Figure 1. µXRF Cd, P and S mapping of a wild type cell cultivated in TAP medium exposed to 70µM of cadmium during 48 hours. E= 3.57 KeV, step-size= 0.2 µm, counting time= 0.3 s/pixel.

When the wild type strain was subjected to an important cadmium stress (growth decrease) in modified TAP medium, cadmium localization changed. Cadmium and phosphorus occupied a large part of the cell (Figure 2), maybe in the choloroplast. A big rich-spot in sulfur and cadmium could correspond to the pyrenoïd. For the speciation, cadmium was predominantly bound to oxigen ligands in the whole biomass (bulk XANES, Figure 5). Thus, in toxic conditions, thiolated compounds are not the main mechanism against cadmium stress. However, µXANES into the cell (spot 1 and 2, Figure 5) showed a percentage of S-ligands higher than bulk XANES suggesting the occurrence of various mechanisms.

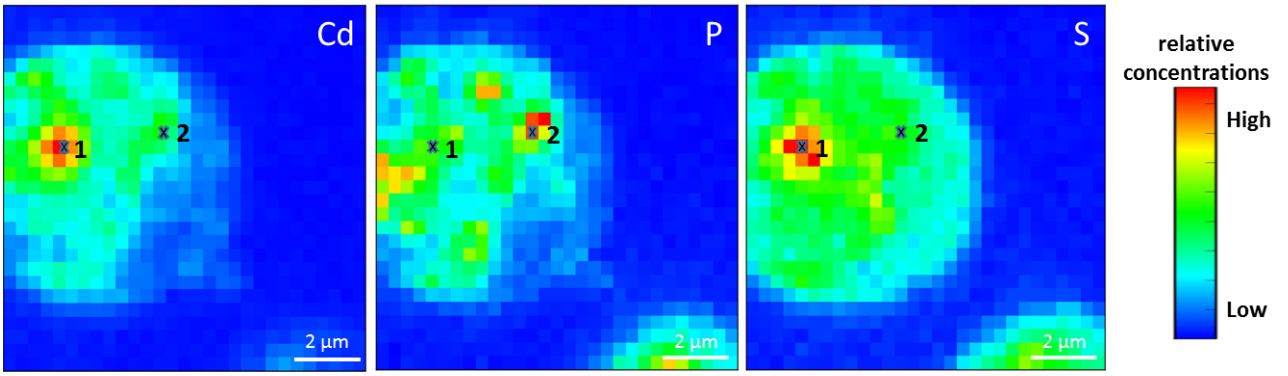

Figure 2. µXRF Cd, P and S mapping of a wild type cell cultivated in modified TAP medium exposed to 70µM of cadmium during 48 hours. E= 3.57 KeV, step-size= 0.5 µm, counting time= 0.3 s/pixel.

For the pcs1 strain, cadmium, phosphorus and sulfur were co-located in a large part of the cell and in a hot spot (Figure 3). The large part could be the chloroplast and the hot spot could be the pyrenïod, into the chloroplast. Concerning the cadmium speciation, bulk analysis (Figure 5) showed cadmium was bound to a mix of sulfur/oxygen (60/40) ligands. Moreover, µXANES on spot 1 and 2, indicated that cadmium was bound to sulfur ligands. Over-expression of phytochelatin synthase into the chloroplast could explain the sulfur localization and the cadmium speciation.

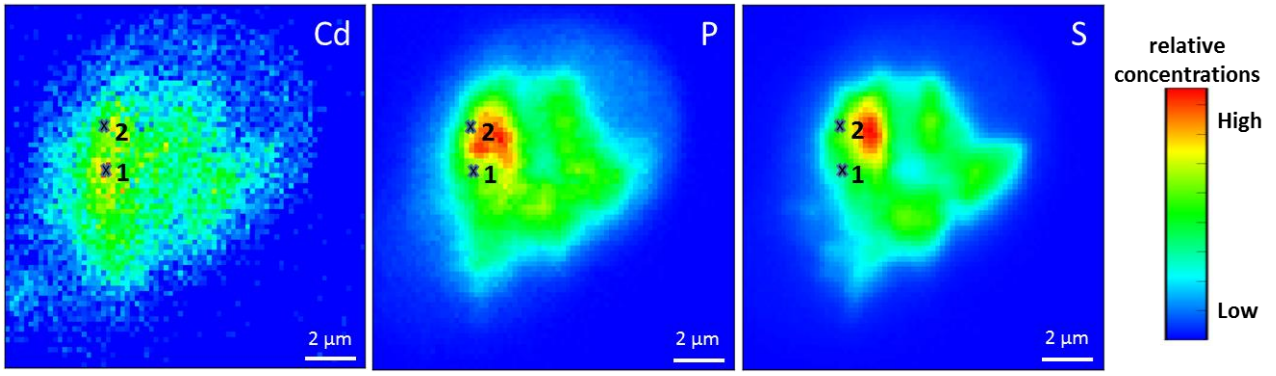

Figure 3. µXRF Cd, P and S mapping of a pcs1 cell cultivated in TAP medium exposed to 70µM of cadmium during 48 hours. E= 3.57 KeV, step-size= 0.2 µm, counting time= 0.2 s/pixel.

For the cw15 strain, cadmium and phosphorus were co-located in large spots (Figure 4). The elemental repartition in cw15 strain seemed to be the same than in wt strain cultivated in TAP medium. Concerning the cadmium speciation, bulk analysis showed that cadmium was bound to sulfur ligands as in wt strain (Figure 5). Cadmium was not enough concentrated to do µ-XANES and very close to the detection limits for the µ-XRF. The weak cell-wall of the cw15 strain did not facilitate the cadmium uptake. The cell-wall could be necessary for the metal uptake.

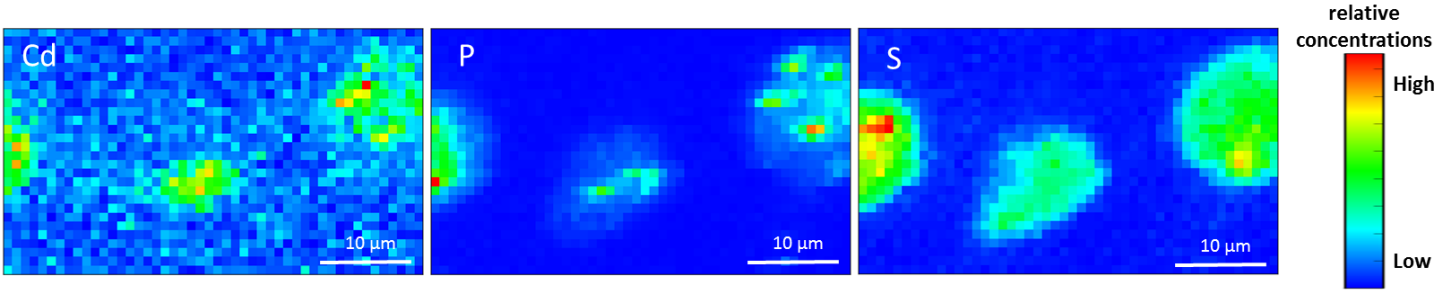

Figure 4. µXRF Cd, P and S mapping of a cw15 cell cultivated in TAP medium exposed to 70µM of cadmium during 48 hours. E= 3.57 KeV, step-size= 0.5 µm, counting time= 0.7 s/pixel.

In conclusion, in wt strain, cadmium was predominantly bound to sulfur ligands in non toxic conditions and to oxygen ligands in toxic conditions. This change is probably related to the cadmium toxicity. In the pcs1 strain, cadmium seemed to be sequestrated by sulfur ligands into the chloroplast and the cw15 strain showed that cadmium needed to be adsorbed on the cell-wall to penetrate the cell. µXRF mapping will be compared to TEM-EDX elemental mapping and cellular fractionation in order to identify cadmium localization in subcellular compartments (organelles).

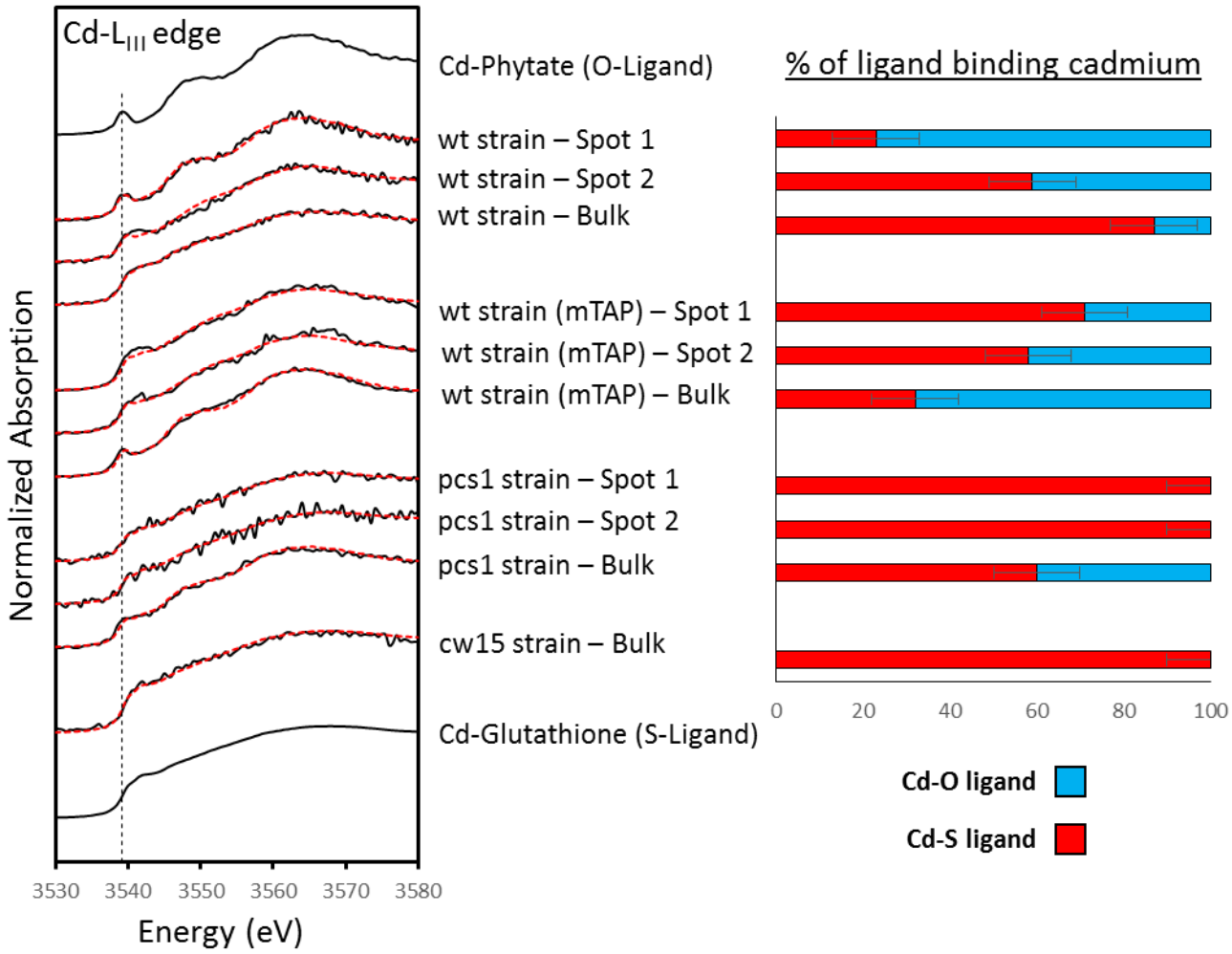

Figure 5. Cd L_{III}–edge XANES spectra collected on bulk *Chlamydomonas reinhardtii* wt, pcs1 and cw15 strains cultivated in TAP or mTAP medium exposed to 70 µM of cadmium during 48H. Cd L_{III}-edge µXANES spectra collected in Cd rich-spots of wt, pcs1 and cw15 strains cultivated in TAP and mTAP medium exposed to 70 µM of cadmium during 48H, compared to Cd references: Cd-phytate as representative of Cd-O bonds and Cd-glutathione as representative of Cd-S ligands. Each spectrum (black lines) is shown with its linear combination fit (red dotted lines). Distribution of Cd species are presented for the samples after normalization of the percentages to 100%. The uncertainty is estimated to ±10%..