Possible biomineralisation of uranium in *Lemna gibba* G3

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Abstract. We investigate biomineralisation of U(VI) accumulated in *Lemna gibba* G3 under laboratory conditions. Almost 50.3±11.2 % of uranium was eluted from biomass resulting into 243.5±111.7 mg Kg^{-1}, and 308.9±189.3 µg L-1 in eluates in a 30 minutes deportation experiment. No further uranium losses or concentration increase in aliquot were observed in weekly analysis of soaked biomass for 5 weeks. Phase Contrast and Scanning Electron Microscope shows crystal formation in and on the fronds. Energy dispersion X-ray showed that the crystals contain uranium and elements that support the phenomenon of metal oxalate formation in Lemna sp. Uranium was likely fixed culture as uranyl oxalates species in *Lemna gibba*. Hence, further studies are required to determination uranyl oxalates species structures and ascertain the biomineralisation.

Introduction

Acceptance of phytoremediation as an alternative restoration technology of contaminated surface waters in abandoned uranium mining sites depends on ability to manipulate the uranium immobilisation capacity and durability. To engineer the phytoremediation capacity requires insight knowledge of the process involved. One of the processes that result into long term fixation of metals in plants is biomineralisation of metals. The term biomineralisation characterizes the formation of inorganic solids by living organisms (Mann et al. 1989). Processes of biomineralisation are either under strict biological control or induced by biological activities leading to adventitious precipitation (Mann et al. 1989). Hence, biomin-

eralisation in remediation of metals in the aquatic system would present one of the most durable fixations of uranium in an ecological way.

In view of this, we investigated uranium immobilisation by *Lemna gibba* G3 through possible biomineralisation processes in synthetic mine water in the laboratory. The investigation were aimed at proving the hypothesis that durability of uranium fixation in *Lemna gibba* and other aquatic macrophyte is partially a result of biomineralisation processes which start with precipitation of uranium-organic complexes e.g. oxalate ligands in the plant and in the milieu media, followed by sedimentation (digenesis). The current hypothesis was reached following our earlier studies, in which we found that *Lemna gibba* exuded low molecular weight organic acids most likely oxalic acids into the media under phosphate deficiency, and also under uranium loading (Mkandawire; Dudel 2005: Mkandawire et al. 2004). Chemical speciation modelling with PhreeqC predicts that complexes of uranyl oxalates are likely to form and precipitated the medium.

There are also publications that document production of calcium oxalates in the fronds of most *Lemna* sp (Kostman et al. 2001: Mazen et al. 2004: Volk et al. 2002). Recent studies have also shown that oxalate production by plants and fungi can result into precipitation of metals inform of insoluble metal oxalates (Ganesh et al. 1999: Lytle et al. 1998: Mazen; El-Maghraby 1998). Oxalates are listed among the low molecular weight organic compounds that are involved in the initial stages of metal biomineralisation by plants (Table 1) (Mann et al. 1989).Thus, the precipitation of uranyl oxalates and actual bioaccumulation of uranium in fronds were assumed to be prime responsible for uranium immobilisation in *Lemna gibba* culture. Other well-known biomineralisation processes of uranium include biological reduction of U(VI) to insoluble U(IV) by microbes (bacteria) involved in sulphate and iron reduction (Benders et al. 2000: Liu et al. 2002: Robinson et al. 1998).

Therefore, it is expected that uranium-oxalate in *Lemna gibba* or particulate-bound uranium should lead to formation of secondary uranium minerals in the sediments of the aquatic system. Once this is achieved, phytoremediation would

Table 1. The type of the inorganic solids (biominerals) found in macrophytes and algae as reported in literature (after Mann et al 1989) relevant for uranium complexation (Hence, Si is not considered.).

Mineral	**Formula**
Calcium carbonate	
Calcite	$CaCO_3$*
Amorphous	$CaCO_3 \bullet nH_2O$
Metal oxalates	
Whewellite	$CaC_2O_4 \bullet H_2O$
Weddelite	$CaC_2O_4 \bullet 2H_2O$
Group IIA metal sulphates	
Barite	$BaSO_4$
Iron oxide	
Ferrihydrite	5Fe2O3•9H2O

*a range of Mg-substituted calcites are also formed

provide a permanent fate of mobile uranium in aquatic systems. Hence, the current study was to confirm the formation of uranyl oxalate complexes in both *Lemna gibba* and it culture media.

Material and Methods

The hydroponic culture and experimental conditions

A strain of *Lemna gibba* G3 was obtained from LemnaTech GmbH (Würselen, Germany – see acknowledgements) and used in the experiment. The strain was cultured in synthetic mine water in semicontinuous mode in ecotron (Plant-growth chamber NEMA GmbH, Netzschkau, Germany) for 21 days (Mkandawire; Dudel 2005: Mkandawire et al. 2004). The synthetic mine water was composed of 5× diluted Hutner media, and other relevant values of water quality of surface mine water in abandoned uranium mines at Lengenfeld and Mechelgrün in the Free State of Saxon, Germany (Mkandawire; Dudel 2005: Mkandawire et al. 2004). These water quality values included 1000 $\mu g\, l^{-1}$ uranium prepared from $UO_2(NO_3)_2 \cdot 6H_2O$, pH r6.5±1.5, Eh range 350 – 550 mV, conductivity of 600, alkalinity or hardness (8.2±2.0 mg L^{-1} dissolved HCO_3^-).The pH was adjusted by drop-wise addition of either 50 mg l^{-1} $NaHCO_3$ or with 65% HNO_3, while water hardness was adjusted with addition of $CaCO_3$. All reagents were of analytical grade.

The ecotron was also programmed to summer conditions of the two abandoned uranium mining sites as follows: 16 h daylight and 10 h dark; 85–125 $\mu E\, m^{-2}\, s^{-1}$ light intensity (PAR) range; and 24±2°C and 16±2°C day and night temperatures, respectively. Chemical speciation was calculated regularly using the PhreeqC geochemical modelling program (Version 2.8, USGS, USA) to ensure optimal solubility and bioavailability of uranium at all time during the culture period. All *Lemna gibba* biomass was harvested after the 21 day of culturing. A few fronds were selected at random for analysis with Scanning Electron while the rest of the biomass was freeze-dried (Christ freeze dryer ALPHA 1-2/LD, Osterode am Harz, Germany) in preparation for the leaching experiment.

Leaching experiment

Approximately 5 g of the freeze-dried biomass were put into Erlenmeyer glass flask containing 500 ml of distilled water. After 10 minutes of stirring with a magnetic stir, 1 ml of water sample and 1 g of biomass were collected, and centrifuged before analysis with ICP-MS (PQ2+ Thermo, Cheshire, England, UK). The procedure was repeated every week for four consecutive weeks. All experiments were replicated four times in factorial arrangement. All biomass samples were

dried in a vacuum drier at 49°C until constant weight was reached. Then, the dry biomass were digested using HNO_3–H_2O_2 mixture in a microwave digester (MW-Digestion, CEM MARS 5, Matthews, North Carolina, USA) in preparation for uranium determination with ICP-MS.

Microscopic analysis

The fronds selected randomly soon after harvesting were divided into samples to be analysed under Phase Contrast Microscope (PCM) (Interference Microscope Peraval Interphako, Carl Zeiss, Jena. Germany), and Scanning Electron Microscope (SEM, JEOL JSM-T330A, JEOL Ltd., Tokyo, Japan),). The samples for PCM were first separated into root-like structures. Then, the root-like structure and leaflets were grounds using separately before being mounted on microscope glass slides. A litre of the medium was also collected at the end of the cultures period and re-concentrated by dewatered in freeze drier i.e. the 1 L of medium was sublimated until the volume was reduced to 20 ml. A drop of the concentrated medium was also mounted on the microscope slides for analysis.

Frond sample for SEM analysis were deep in liquid nitrogen for rapid freezing to avoid ice development during slow freezing, which damages the cells and cell components. The samples were held on copper holder, and freeze-dried (EMITECH K1250, Ashford, Kent, UK). Then, the samples were broken before being exposed to carbon steam (EMITECH K-450X Carbon Evaporator, Ashford, Kent, UK). After carbon steaming, the samples were investigated for any crystal formation on Scanning Electron Microscope. An Energy Dispersive X-ray (EDX) spectrograph coupled to the Scanning Electron Microscope set at 15 kV was used to determine micro-chemical composition of the crystals.

Results

The leaching and decay experiment was conducted to determine the amount bioaccumulated uranium which readily re-mobilised once dry matter get into contact with water, and when the biomass degrades. The remobilised uranium was collected in the aliquot, and the durably fixed uranium remained in the *Lemna gibba* biomass. The results of analysing the aliquot and biomass samples collected weekly for a period o five weeks (figure 1). It was observed that uranium biomass initially lost about 50.3±11.2 % or the accumulated uranium before stabilising within the first week. In the following weeks, loss of uranium from the biomass was negligible. Likewise, after dissolution of uranium into the aliquot in the initial stage, there were no significant observable increases in uranium concentration in the aliquot until the end of the experiment. Thus, uranium fixation in the biomass remained the same by the time of termination of the experiment. *Lemna gibba* biomass has not degraded by the end of the experiment.

The PCM was used to identify crystal-like structures in *Lemna gibba* in relation to uranium in the media. The results of PCM analysis showed that there is formation of crystals more in the leaflets, followed by root-like structure and little in the solution (Figure 2). In the leaflets, crystals were observed in both control and uranium containing experiments. The only difference between the experiments was that the crystal formations as observed in uranium experiment were bigger and more than in the control experiment (Figure 2 (a) and (b)). Crystals were also observed in the root-like structure of *Lemna gibba*. However, it was distinctly more crystal formation in the roots of Lemna gibba exposed to uranium (Figure 2 (c) and (d). No crystals were observed in the media of the control experiment, while some crystals were observed in the uranium experiment. There may be a number of reason for low formation of the crystals in the media like the ration between the biomass and the media, and also dissolution of crystals in the media, just to mention a few.

The purpose was of SEM examination to substantiate presumed uranyl oxalate complexes based on crystal identification with PCM, and to determine localisation of uranium in *Lemna gibba* fronds. Using the SEM-EDX facility, the analysis was expected to ascertain that the crystals observed contained the uranium. The pictures of the observation and localisation of the crystals presumed to be the uranyl oxalate in and on the surface of *Lemna gibba* are presented in Figure 3. Figure 3 (a) indicates that the formations of the crystals are in the proximity of the *Lemna gibba* cell wall.

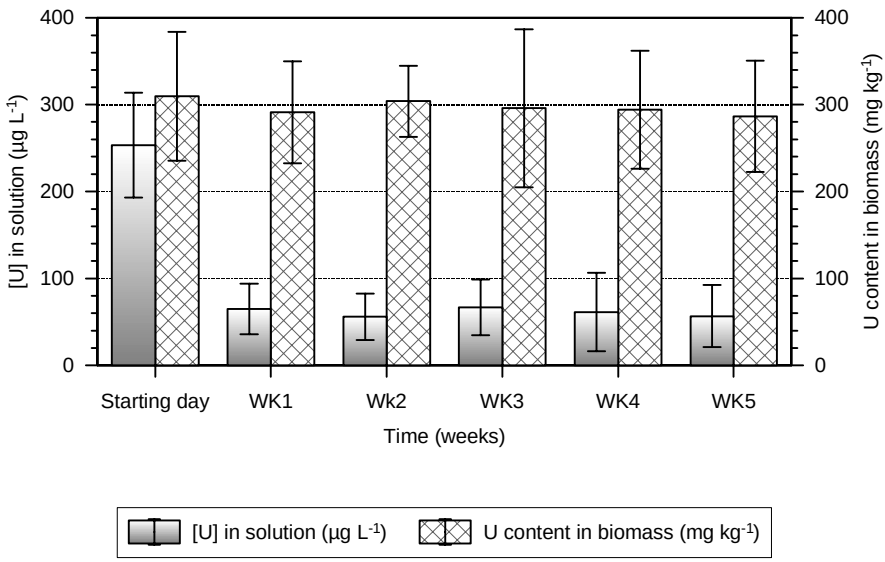

Fig.1. Fractions of uranium in fronds and concentration of elutes and leachates after elutions and leaching of *Lemna gibba* dead biomass. The analyses on the starting day were done on the after eluted biomass and the resulting eluent. The values are mean of four replications and the error bar is standard deviation.

The mean size of the crystals in the cells is 40±20 µm (Figure 3(b)). On the surface of *Lemna gibba*, the crystals are also observed. Figure 4 presents the analysis of the element composition of the crystals using the EDX coupled to SEM. The results show that the crystals contain carbon, hydrogen and oxygen, which are components of oxalates. Further, the crystal contained large amount of uranium and calcium. Some of Fe was observed. Other components like K, S and Cl are expected in the plant material because they are essential elements.

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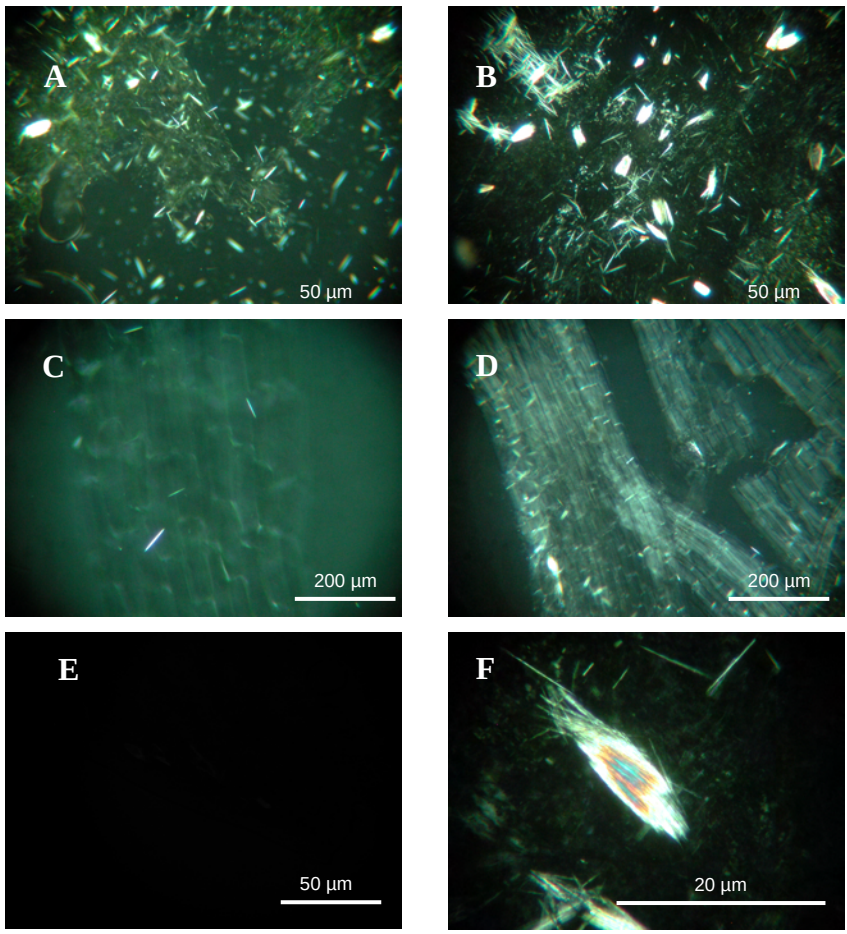

Fig. 2. Crystals in and on the surface of *Lemna gibba* and its media under Phase Contrast Microscope. (A) and (B) are crystal in the frond leaflet under control and 1 mg L^{-1} initial uranium in the media, respectively; (C) and (D) are crystal in the frond root under control and 1 mg L^{-1} initial uranium in the media, respectively; and finally (E) and (F) are crystal in the media under control and 1 mg L^{-1} initial uranium in the media, respectively.

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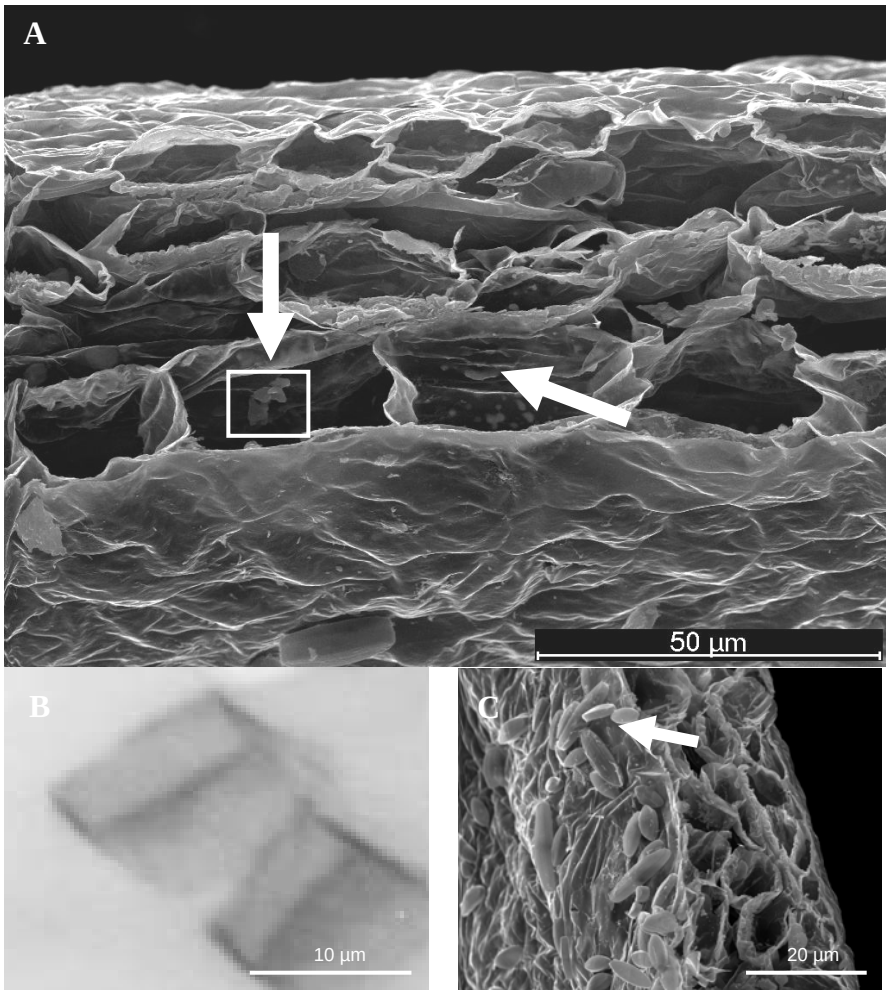

Fig. 3. Scanning electron microscope pictures of *Lemna gibba* fronds. (A) shows crystal development in the cells; (b) is the higher magnification of the crystals and the part analysed by Energy Dispersive X-ray (EDX); and (C) shows some crystal formation on the surface of *Lemna gibba* leaflet.

Discussion

There are a few documentations of saccharides, proteins, peptide, and oxalate formation in response to toxic stress in *Lemna* sp. in literature (Amado et al. 1980: Farooq et al. 2000: Horner et al. 2000: Kostman et al. 2001: Mazen; El-Maghraby 1998: Mazen et al. 2004: Ovodova et al. 2000: Sauter 1988). One of the recent and detailed work come from Mazen et al (2004), who described the formation of oxalate crystal are in crystal-forming cells (crystal idioblasts). In the current study, we have found the crystal similar to the structure described by Mazen et al (2004) (figure 3). Additionally, the structures are also found in the submerse root-like structures and even in the media (figure 2). Some studies have attribute formation of calcium oxalate in *Lemna* sp. as sinks for bulk regulation of calcium, and detoxification of oxalic acid. The association of the crystals with uranium found in our study may indicate that the crystal formation may be general compartmentalisation or intracellular sequestration of toxic substances in the plants. Immobilization of metals can result from sorption to cell components or exopolymers, transport and intracellular sequestration or precipitation as organic and inorganic compounds, e.g. oxalates, and sulphides. Oxalate forms complexes with a wide variety of metallic ions including transition metals (e.g., Fe^{3+} and Al^{3+}) and actinides (e.g., UO_2^{2+} and PuO_2^{2+}) (Ganesh et al. 1999: Mazen; El-Maghraby 1998). However, uranium observed in oxalate crystal idioblast, in this study, has never been described before (figure 2 and figure 3). Similarly, formation of uranyl oxalate crystal (figure 2) confirms modelling results in our previous study (Mkandawire and Dudel, 2002). The durability of fixation demonstrated by the leaching experiment confirms also that uranium might be tightly fixed in the biomass (figure 1). In atomic energy industries, precipitations of uranyl oxalates

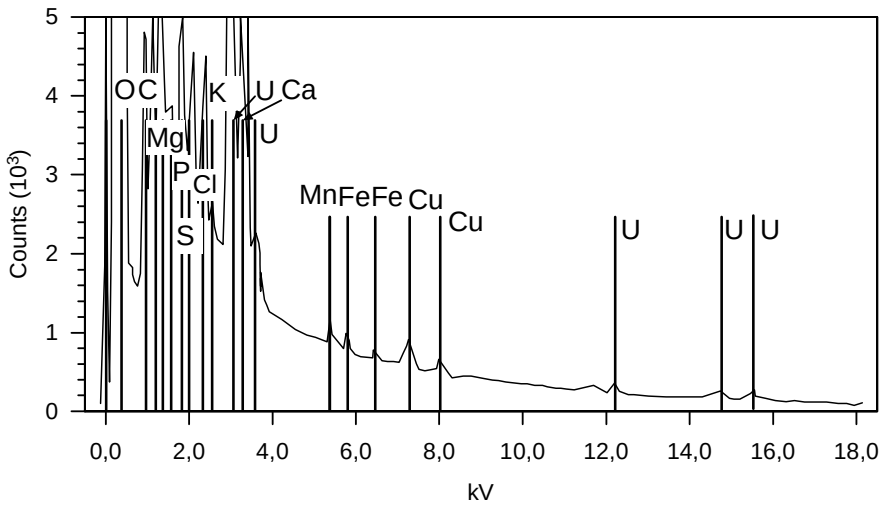

Fig. 4. Energy Dispersive X-ray (EDX) spectrograph of crystal shown in Figure 3 (a).

are used to separate and purify uranium in fuel reprocessing flow sheets. Oxalates are also used to concentrate uranium in alkaline waste solutions by forming insoluble uranyl oxalate precipitates (El-Nadi et al. 2003: Robinson et al. 1998). Hence, if oxalate form naturally in *Lemna gibba* as shown in this study, biomineralisation of uranium in surface mine waters is possible.

A number of synthetic polycrystalline uranyl oxalate complexes have also been investigated (Havel et al.: Szabó; Fischer 2002: Tel 1999). Based on the empirical formula of the compounds and analytical results, structures of the complexes have been identified as linear uranyl cations connected by tetradentate bridging oxalate groups yielding a two-dimensional network. These nets are stacked to form the crystal structure. Such structures includes $UO_2C_2O_4 \cdot 3H_2O$, $(UO2)_2C_2O_4(OH)_2 \cdot 2H_2O$, $K_2(UO_2)_2(C_2O_4)_3 \cdot 4H_2O$ or $Ca(UO_2)_2(C_2O_4)_3 \cdot 4H_2O$ which form at room temperature as determined by the research group of Dr Burns at University of Notre Dame in USA (personal communication with Paul Giesting). Comparison of these synthetic crystals with those produced natural have a number of similarities such as the colour (yellow), size and shape. Nonetheless, the crystals from *Lemna gibba* need to be further investigated to ascertain if they are really uranyl oxalate species, and identify the real chemical structure.

Conclusion

The current results from our experiments indicate that uranium is probably immobilised *Lemna gibba* in aquatic system as crystal-like complexes most likely uranyl oxalates species (e.g. $UO_2C_2O_4 \cdot 3H_2O$, $(UO2)_2C_2O_4(OH)_2 \cdot 2H_2O$, $K_2(UO_2)_2(C_2O_4)_3 \cdot 4H_2O$ or $Ca(UO_2)_2(C_2O_4)_3 \cdot 4H_2O$) in and on fronds, and in the media through exudation. With the knowledge that metal oxalate end into biomineralisation, and the results obtained in this study on stability of uranium in decaying organic matter, *Lemna gibba* facilitate significantly the biomineralisation of uranium. If this happens, then uranium immobilisation using macrophytes like *Lemna gibba* would present a durable and clean technology of phytoremediation of contaminated surface water in abandoned uranium mines. Further investigations are required to determine of the structure degree of order of the crystals other possible minerals U complexes and qualification per volume biomass unit inside and outside of the cells and in the medium in relation to the environmental conditions of the uranyl oxalate crystals in *Lemna gibba*. It would therefore imperative to identify the structure using X-Ray Diffraction or similar technology.

Acknowledgement

The research was supported by BMBF project grant No. 02WB0222. Ernst Bäucker (Institute of Forest Utilisation, Dresden University of Technology) guided analytical determination with SEM-EDX facility. Arndt Weiske did ICP-

MS analyses. Dr. Joachim Rotsche assisted in mineral crystal identification with Phase Contrast Microscope. The strain of *Lemna gibba* G3 was kindly provided by Matthias Eberius of LemnaTech GmbH. Paul Giesting of Environmental Molecular Science Institute at the University of Notre Dame in USA kindly provided structures of synthetic uranyl oxalate species for comparison with those found in *Lemna* culture.

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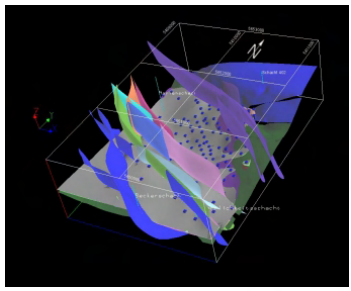

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