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Spectroscopic investigations of U(VI) species sorbed by the green algae *Chlorella vulgaris*

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Abstract The green alga *Chlorella vulgaris* has the ability to bind high amounts of uranium(VI) in the pH range from 3 to 6. At pH 3 up to 40% of the uranium are bound by the algal cells. The uranium removal is almost complete at pH 5 and 6 under the given experimental conditions. Scanning electron microscopy and laser-induced fluorescence spectroscopy were used to characterize uranyl species formed in the selected pH range. The micrographs show a regular distribution of U(VI) on the cell surface. Fluorescence spectroscopic investigations of formed algal uranyl complexes indicate that the binding of U(VI) to carboxyl groups plays a dominating role at pH 3, whereas a minor impact of organic phosphate compounds on the U(VI) sorption cannot be excluded. In contrast, at pH 5 and 6 the phosphate groups are mainly responsible for the removal and binding of U(VI) by formation of organic and/or inorganic uranyl phosphates.

Keywords Uranium(VI) · Green algae · Sorption · Complexation · TRLFS · REM-EDX

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Introduction

The green algae (Chlorophyta) are a group of aquatic "low" plants, mostly living in fresh water, in salt and/or brackish water, in soils and in tree trunks. Due to the ubiquitous occurrence of algae their influence on the migration processes of uranium and other actinides in biological and geological environments is of fundamental interest. In addition, algae play an economically relevant role as food as well as in food additives, such that heavy metals and actinides taken up by algae can make their way into the food chain of humans and pose a health threat.

Chlorella species are known to possess high binding capacities for heavy metals like copper, nickel, cadmium, zinc and lead. This accumulation is dependent on the concentration of the biomass, the pH value, the temperature and the cultivation time (e.g., Maeda et al. 1990; Harris and Ramelow 1990; Cho et al. 1994; Sandau et al. 1996; Lau et al. 1999; Wong et al. 2000; Gin et al. 2002; Tien 2002; Mehta et al. 2002; Franklin et al. 2002; Chu and Hashim 2004; Fraile et al. 2005; Abu Al-Rub 2006; Aksu and Donmez 2006). Some studies about the sorption behaviour of the unicellular microalgae *Chlorella* regarding uranium(VI) have been published. Horikoshi et al. (1979a) reported that the uptake of uranium by *Chlorella regularis* is very rapid and is not affected by light, temperature, or treatment with metabolic inhibitors. The results indicate that the uptake of uranium by *Chlorella* is only dependent on the

physical adsorption on the cell surface. Electrostatic attraction and chemical sorption of uranyl ions through complex formation with cellular ligands thus play a role in this process. In the same year Horikoshi published results about the determination of uranium in the *Chlorella* cells and the cell walls using neutron activation analysis (Horikoshi et al. 1979b). Dry or hot-water treated cells are the most efficient cells for uranium recovery from the aqueous systems. About 34% of uranium taken up by dry cells was bound by the cell wall and in case of living cells, 85% of uranium was determined in the wall. Furthermore, they could show that the *Chlorella* cells grown under various culture conditions have the same ability to accumulate uranium. The largest amount of uranium was determined in cells grown under autothrophic conditions and which were heat-killed in comparison to living cells (Horikoshi et al. 1981). Nakajima et al. (1979) found that the uranium uptake is hindered by phosphate and carbonate ions in the solution, forming stable uranyl phosphate and carbonate complexes, which will not be taken up by *Chlorella* cells. Additionally, the uptake was not affected by cations (Na, K, Mg, Ca, Mn, Co, Ni, and Zn), nitrates, sulphates and thiosulphates. This suggested to them that uranium was taken up as UO_2^{2+} or UO_2OH^+ in exchange for protons of organic ligands. Singhal et al. (2004) postulated that in dependence on the dissolved species different functional groups of the *Chlorella* cells are involved in the uranium binding. They formulated that neutral uranyl carbonate species such as (UO_2CO_3) may adsorb on the algal cells while negatively charged species $(UO_2(OH)_3)^-$ may bind to different functional groups, including carboxyl, amine, hydroxyl and phosphate. No explanation was given how negatively charged uranyl species may bind to deprotonated forms of these functional groups. In addition, the possible impact of the calcium alginate itself on the uranium immobilization was not investigated and not discussed. For instance, uranium may form a stable carbonate complex with calcium (Bernhard et al. 2001) and/or may be bound by carboxyl and hydroxyl groups of the alginate.

The aim of our study was to characterize the molecular structure of the U(VI) complexes formed on the *Chlorella vulgaris* cells using time-resolved laser-induced fluorescence spectroscopy (TRLFS). To our knowledge, this is the first time that a spectroscopic method was used to investigate the uranium binding form in algae–uranyl complexes and in the corresponding aqueous solutions. After the sorption process, differences between the uranium speciation in the solutions and in the biomass should be detected at different pH values. Sorption experiments at different initial uranium concentrations, amounts of biomass and pH values precede these spectroscopic investigations. Additionally, the uranium distribution on the cell surface was visualized using scanning electron microscopy.

Materials and methods

Cultivation and characterization of algal cells

Chlorella vulgaris was grown in a liquid algal full medium (Esser 2000) under air supply and light until the end of the exponential growth phase. The algal biomass was harvested by centrifugation and washed with 0.9% (weight per volume, w/v) sodium perchlorate solution. The purity of algal culture was verified by means of light microscope. The cells were stored at −20°C.

Sorption experiments

The concentration of resuspended algal biomass in each reaction solution was 0.125 g dry mass/l and 0.750 g dry mass/l for two parallel serials. For the sorption experiments the algal biomass was contacted 72 h or 144 h with U(VI) solutions at pH 3, 5 and 6. The uranium concentration varied in the range from 1×10^{-5} M to 3.7×10^{-4} M. The experiments were performed in 0.9% (w/v) $NaClO_4$ solution. The pH values of the solutions were adjusted with $HClO_4$ or NaOH. The uranium concentration in each solution was checked by ICP-MS analyses (Elan 9000, Perkin-Elmer) after separation of the algal biomass. The amount of sorbed U(VI) in the biomass was calculated by formation of the difference between initial and final concentration of uranium in the solution. In addition, equal samples without biomass were investigated.

SEM and EDX microanalysis

Scanning electron microscopy investigations (SEM) were carried out with a high-resolution Hitachi

S-4800 microscope. Secondary and backscattered electrons were used for the detection applying acceleration voltages of 1 kV (SE) and 10 kV (BSE). The chemical composition of single points (minimum size 1–2 μm) or areas of the uranium-containing depositions on the algal cells were determined with an energy dispersive X-ray (EDX) microanalysis system INCA (Oxford Instruments). This system employed an integrated Si-detector and S-UTW-window. Depth information up to several micrometer can be obtained. Details of the method are described in Schmidt et al. (1994).

The uranium containing algal samples were washed with 0.9% (w/v) sodium chloride solution (pH 7.5) and were fixed in 4% (w/v) glutaraldehyde (pH 7.5) for 24 h at 4°C. The cells were then washed again with 0.9% (w/v) sodium chloride and were dehydrated by washing with rising ethanol concentrations. For the SEM- and EDX-measurements the dehydrated samples were fixed with sticky conductive carbon tapes on the sample holder. The samples were spattered with carbon for a better conductivity.

TRLFS

A Nd-YAG laser (INLITE, Continuum) with laser pulses at 266 nm and an average pulse energy of about 250 μJ was used for laser-induced fluorescence measurements. The emitted fluorescence light from the initial solutions and the algal samples was detected using a 270 nm spectrograph (EG & G Princeton Applied Research; model 1235 Digital Triple Grating) in combination with a CCD camera (Princeton Instruments, INC./Roper Scientific INC). The resulting spectra were measured in time-resolved mode using an internal digital delay generator. To correlate the laser output with the start of the measurement an external delay generator (Stanford Research system, INC., model DG535) was used. The TRLFS spectra from 349.8 to 663.6 nm were recorded by averaging 100 laser pulses using a gate time of 2 μs. Each sample was measured 3 times on every delay time. All functions of the system were computer controlled using the software WinSpec 32 which was provided by Princeton Instruments. A more detailed description of the equipment is given in Geipel (2006). The fresh and washed algal samples of the second sorption experiments were used for the detection of uranium(VI) species carrying out fluorescence emission spectroscopic measurements.

Results and discussion

Calculated U-speciation in the initial solutions

The calculation of the U(VI) speciation with the computer program EQ3/6 (Wolery 1992) using NEA data base (Guillaumont 2003) for 1×10^{-4} M initial uranium solutions showed that the uncomplexed uranyl ion UO_2^{2+} dominates the U(VI) speciation at pH 3. At pH 5, uranium forms in addition to the free uranyl cation (18.9%) different hydroxides like $(UO_2)_3(OH)_5^+$ (45.8%), $(UO_2)_2(OH)_2^{2+}$ (15.8%), UO_2OH^+ (11.3%), $(UO_2)_4(OH)_7^+$ (5.3%) and $(UO_2)_3(OH)_4^{2+}$ (2.2%). At pH 6, the uranyl species $(UO_2)_3(OH)_5^+$ (65.4%), $(UO_2)_4(OH)_7^+$ (18.5%), UO_2OH^+ (2.7%) and $(UO_2)_2CO_3(OH)_3^-$ (10.6%) are the main species in this initial solution. In the broader concentration range of 1×10^{-5} M to 4×10^{-4} M at pH 3 and 6 the uranium speciation in solution is similar to the calculated speciation of the corresponding 1×10^{-4} M uranium solutions. At pH 5 and a uranyl concentration $<1 \times 10^{-4}$ M the speciation changes in favour of the free uranyl cation (up to 49%) and UO_2OH^+ (up to 30%).

Biosorption

At pH 3 and a contact time of 72 h low uranium amounts were removed from the solution and sorbed by algal cells (0.125 g algae dry weight/l; 1×10^{-4} uranium) (Fig. 1a). Only 8.9% of initial uranium was bound to the biomass. In contrast to that, 87.4% of the uranium in the initial solutions was immobilized by algal cells at pH 6. Furthermore, an increase of the binding capacity with longer contact time and higher pH value was determined. The increasing deprotonation of the functional groups of the algal cells, in particular carboxyl and phosphate groups, with increasing pH value should be the reason for this.

A higher binding rate for uranium was determined during the sorption experiments with a biomass concentration of 0.75 g algae dry weight/l, 1×10^{-4} M uranium and the same contact time of 72 h in comparison to the corresponding experiment with a lower algal concentration in the initial

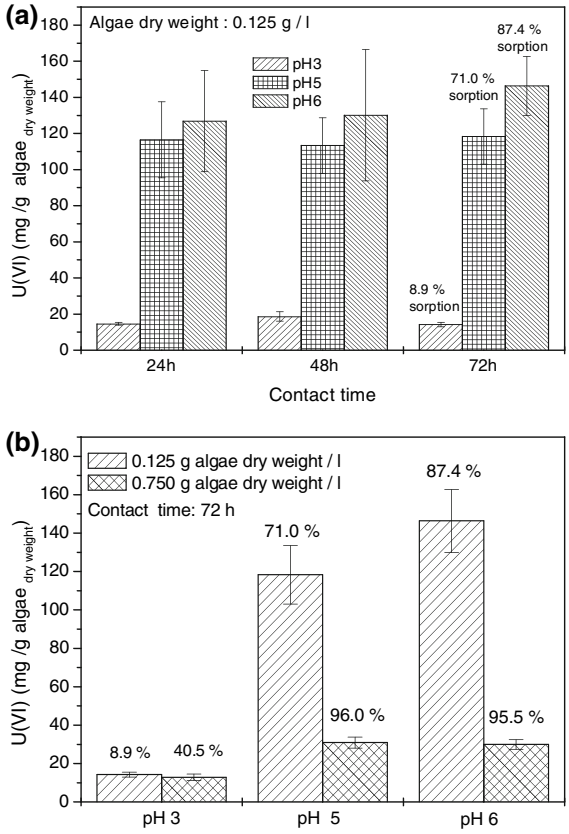

Fig. 1 Total amount of uranium(VI) removed from the initial solution ($[U]_0$: 23.8 mg/l (1×10^{-4} M)) by algal cells as a function of (**a**) contact time and pH value and (**b**) algal concentration and pH value

solution (Fig. 1b). Approximately 40% of initial uranium was found in the algal biomass at pH 3. About 96.0% of the uranium in form of positively charged species was bound at pH 5 and 95.5% at pH 6. The sorption process was associated with a small decrease of the pH value in the solution by 0.3 units or less, compared to the initial pH of 5 and 6. The pH value of 3 remained constant. Contrary to the increase of the percentage uranium sorption, a decrease of the binding capacity with increasing biomass concentration could be observed, to a greater extent at pH 5 and 6 than at pH 3, given in Fig. 1b. These findings can be explained by an increase of the number of possible binding sites with increasing biomass concentration.

Uranium biosorption experiments were carried out at various metal concentrations. Beside the algal concentration, the pH value and contact time, the uranium concentration initially present in the solution influences the removal efficiency. Figure 2 shows that uranium is not sorbed linearly with increasing uranium concentration, ranging from 2.38 mg/l (1×10^{-5} M) to 88.4 mg/l (3.7×10^{-4} M) metal. The corresponding percentage uranium removal from the solution by algal biomass decreases with increasing initial U-concentration in the range from 13.1 to 4.9% at pH 3. In case of experiments at pH 5 and 6 the uranium sorption decreases under these experimental conditions from 91.9 and 94.1% to 33.4 and 54.2%, respectively. This fact points to a limited number of binding places in the algal biomass. The change of uranyl speciation in the designated uranyl concentration range at pH 5 is negligible in this process.

The affinity between the algal biomass and uranium could be quantified by adjusting the uranium sorption values to a Langmuir and a Freundlich isotherm. The Langmuir model yields better results (regression coefficients: 0.98 (pH 3), 0.99 (pH 5) and 0.99 (pH 6)) than the Freundlich model (regression coefficients: 0.97 (pH 3), 0.98 (pH 5) and 0.95 (pH 6)). Therefore, in the following the sorption process is described applying only the Langmuir model. The Langmuir isotherm is given by the Eq. 1:

$$C_{Bio} = \alpha \cdot C_{s,e} \cdot C_{Bio\,max}/(1 + \alpha \cdot C_{s,e}) \quad (1)$$

Here C_{Bio} is the uranium concentration in the algal biomass, α the Langmuir constant, $C_{s,e}$ the uranium equilibrium concentration in the solution and $C_{Bio\ max}$ the maximal uranium concentration in the algal biomass. After linearization of the Eqs. 1–2

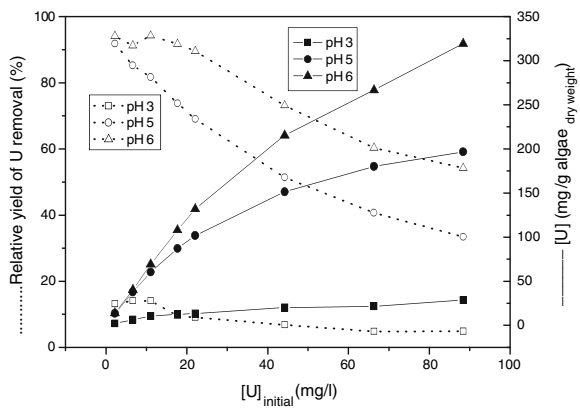

Fig. 2 Uranium(VI) sorption by algal cells as function of $[U(VI)]_{initial}$ and pH value

$$C_{s,e}/C_{Bio} = (1/C_{Bio\,max}) \cdot C_{s,e} + 1/\alpha \cdot C_{Bio\,max} \quad (2)$$

the Langmuir constant is calculated to be $\alpha = 0.033 \pm 0.005$ l/mg. The maximal sorption capacity is $C_{Bio\ max} = 35.7 \pm 3.3$ mg/g at pH 3. By contrast a higher constant and a maximal binding capacity were obtained as a result of experiments at pH 6. They are $\alpha = 0.271 \pm 0.067$ l/mg and $C_{Bio\ max} = 326.8 \pm 16.0$ mg/g. This means, in both systems the binding mechanism and formed uranyl complex species are different. The free uranyl cation, that dominates the U(VI) speciation at pH 3, seems to form less stable complexes with the biomass in comparison to the hydroxide species present at pH 6. At pH 5, the free uranyl ion and several hydroxide species exist in solution and were bound by different functional groups of the biomass. Therefore, the formed complexes differ also in their binding stability. Realizing a better fit by two straight-line segments of the sorption isotherm curve to determine different binding places and complexes was not feasible. Thus a middle maximal binding capacity of $C_{Bio\ max} = 209.2 \pm 7.4$ mg/g and a Langmuir constant $\alpha = 0.181 \pm 0.031$ l/mg was calculated. *Chlorella vulgaris* cells have a higher maximum sorption capacity and affinity for uranium than for Cu, Cd, Ni and Zn at the corresponding pH value (Fraile et al. 2005).

Localization of U(VI) species on the algal cell surface

Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) microanalysis were used to localize and characterize uranium complexes on the surface of algal cells. SEM images of uranium containing *Chlorella* cells are depicted in Fig. 3a and b as examples. The pictures clearly show the spherical cell shape of *Chlorella vulgaris.* Differences in the grey tone reflections by detection of the backscattering electrons indicated differences in the elemental compositions. As obvious from Fig. 3a uranium is regularly distributed on the cell surface. The EDX analysis of selected areas of the cell surface in Fig. 3c showed, additionally to carbon, oxygen and sodium uranium, phosphorus and sulphur as main elements.

Spectroscopic determination of U(VI) species

Figure 4 shows the uranyl luminescence spectra of the contaminated algal biomass in comparison with the uranyl spectra of the solutions before and after the experiment (0.75 g algae dry weight/l, 1×10^{-4} M uranium) at pH 3, 5 and 6, respectively, at a contact

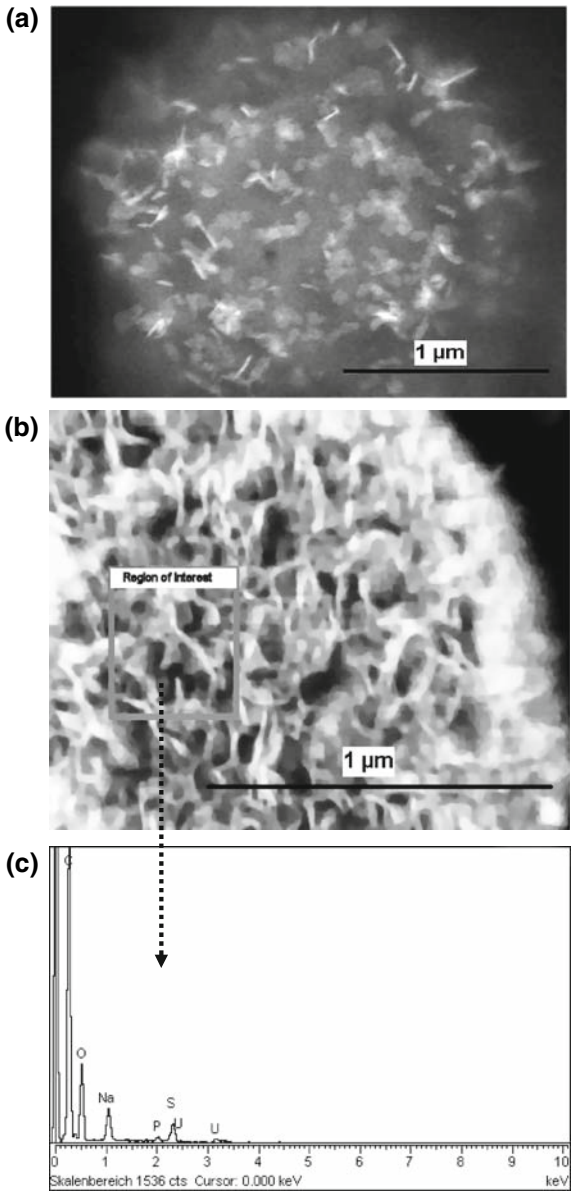

Fig. 3 (**a**) and (**b**) SEM-pictures (BSE) of *Chlorella vulgaris* cell contaminated with uranium at pH 5, coating with carbon, (**c**) corresponding EDX-spectrum of selected area of cell surface

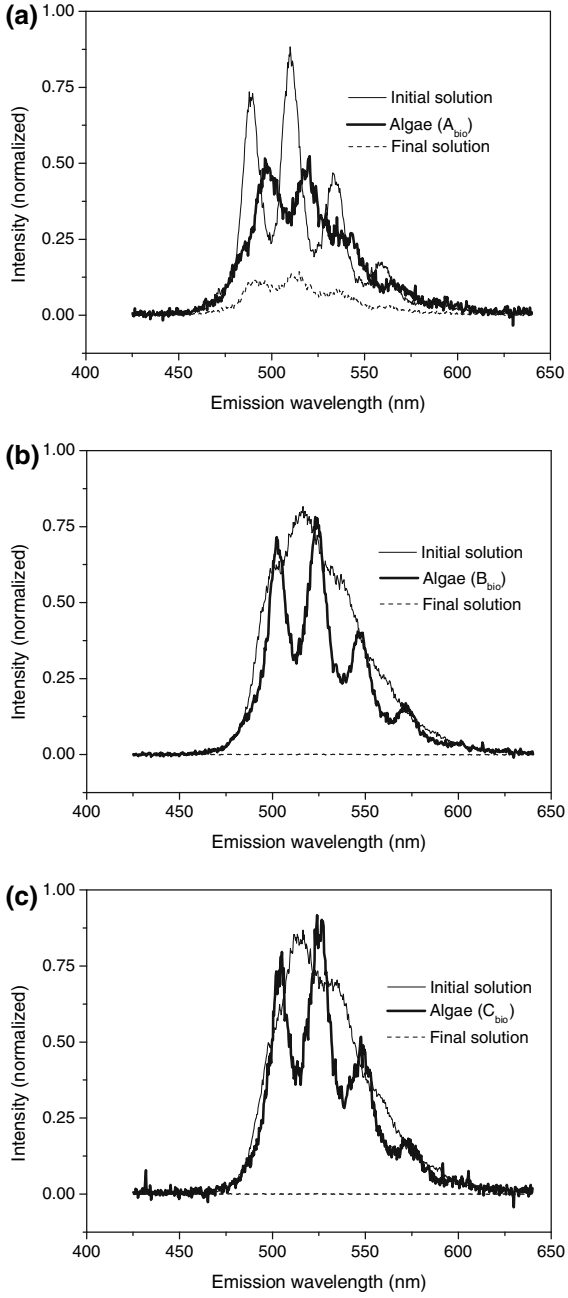

Fig. 4 Luminescence spectra of different samples of the U(VI)/algal system at pH 3 (**a**), pH 5 (**b**) and pH 6 (**c**), initial solution: 1×10^{-4} M U(VI), final solution after 144 h contact time

time of 144 h. The extention of the contact time from 72 to 144 h caused no significant change in the absolute uranium binding and sorption capacity. The variations lie within the error range.

The uranium speciation in the initial solutions was investigated with TRLFS verifying the calculated U(VI) species by determined spectral lines (Fig. 4 and Tables 1 and 2) and lifetimes (not given here). The $(UO_2)_2CO_3(OH)_3^-$ is not detectable at room temperature using TRLFS, due to the quenching of carbonate.

In our experiments, the luminescence intensity of uranium in solution decreases strongly after contact with algae. This can be ascribed to a decrease of the uranyl concentration, a small change of the pH value of the solutions (at pH 5 and 6), and an alteration of the composition of dissolved uranyl species during the sorption processes. ICP-MS analysis demonstrated, that 63.5% of initial uranium is still dissolved after the sorption process at pH 3. But only one third of the uranium in solution is detectable by luminescence spectroscopy, whereas its emission bands are slightly shifting to higher wavelengths. It seems that the remaining dissolved uranium consists not only of the free uranyl ion, but may also form complexes with organic ligands released possibly from algae. Obviously these complexes do not emit any luminescence light and/or luminescence light with a small spectral shift. Furthermore, quenching processes by the organic ligands cannot be excluded. At pH 5 and 6 no luminescence spectrum of the solution after the sorption experiment was obtained, due to too low uranium concentrations. Only 0.6–2.4% of the initial uranyl compounds remain in solution. This means, the luminescence yield is under the limit of detection for the described measurement system. In addition, binding processes shift the equilibrium from residual dissolved species to the free uranyl species (calculated with EQ3/6 and without concideration of possible organic uranyl complexes) that have a lower relative luminescence yield in comparison to those of the uranyl hydroxides.

Uranyl species were detected in the algal biomass of all experiments. That means, uranium remained in oxidation state (VI) after interaction with algal cells. Generally, the determined luminescence lifetimes of the uranyl species in the algal samples are shorter than the lifetimes of the uranyl species in the initial solutions. But they cannot be compared with each other due to the different solvation (number of H_2O molecules) and structural conditions.

The emission maxima of the uranyl species in the algal sample A_{bio}, B_{bio} and C_{bio} are shifted to higher

wavelengths compared to the spectral maxima of the free uranyl ion in the initial solution A and to the spectral lines of the hydroxides in the initial solutions B and C, respectively (see Table 1). This indicates that the uranium speciation in the initial solution and on and/or inside the cells of *Chlorella vulgaris* is different. In addition, differences were found in the spectra of the uranium containing algae biomass A_{bio} and B_{bio} or C_{bio}. A clear identification of the formed uranyl species at pH 3 is difficult. The obtained luminescence spectra are similar to spectra of model uranyl complexes with carboxylic ligands. This means that the carboxylic group is the dominating binding place for uranium on the cell surface and/or in the algal cell at weakly acid sorption conditions. The formation of minor organic complex species with other functionality like phosphate groups is possible and cannot be excluded (e.g., uranyl sugar phosphates, see Table 2). The position of the uranyl emission bands in the spectra of algae at pH 5 and 6 are almost identical and indicate that the same functional groups are involved in the complexation of uranium. The spectra are comparable with those obtained for uranium containing lupine samples (Günther et al. 2003), where uranium is mainly coordinated to inorganic and/or organic phosphate groups (examples in Table 2 and Fig. 5). The determination of the uranyl phosphate species as main species in the algal biomass at pH 5 and 6 support the results of the investigations by scanning electron microscopy, whereas uranium in the immediate vicinity to phosphorus could be localized.

Table 1 Luminescence emission bands of uranyl species in the algal biomass of *Chlorella vulgaris* in comparison to the bands of the sum of uranyl species in the initial solutions

Sample	Emission bands (nm)					
Initial solution (pH 3)	472.3	489.1	510.2	533.3	558.3	586.9
Solution after contact time (pH 3)	471.1	491.5	512.9	535.7	562.2	590.4
Algae (A_{bio})	481.6	497.9	518.5	539.3	565.3	595.7
Initial solution (pH 5)	480.7	497.6	515.0	534.7	557.1	587.4
Algae (B_{bio})	488.7	503.0	523.6	546.5	571.0	598.0
Initial solution (pH 6)	483.4	497.4	513.2	533.2	555.8	585.7
Algae (C_{bio})	488.1	504.0	524.9	547.5	571.7	597.4

Table 2 Luminescence data of different uranyl model compounds and uranium(VI) in other biosystems

Species	Emission bands (nm)						Reference
UO_2^{2+}	471.3	488.9	510.5	533.9	559.4	585.5	Geipel et al. (1996)
UO_2OH^+	480.7	497.3	518.4	541.3	566.4		Eliet et al. (1995)
$(UO_2)_2(OH)_2^{2+}$	481.3	498.3	519.7	543.4	566.7	602.8	Eliet et al. (1995)
$(UO_2)_3(OH)_5^+$	484	498	514	534	557	583	Sachs et al. (2007)
$UO_2(malonate)_2^{2-}$	479	496	517	542	566		Brachmann et al. (2002)
$UO_2(glycine)_2^{2+}$	478.7	495.3	516.7	540.6	565.0	594.4	Günther et al. (2007)
$(UO_2)_x(PO_4)_y$	488.0	503.0	523.7	546.9	572.5	601.3	Brendler et al. (1996)
$Ca(UO_2)_2(PO_4)_2 \cdot 10H_2O$	488.6	504.0	524.2	548.0	573.9	602.4	Geipel et al. (2000)
$Ca(UO_2)_2(PO_4)_2 \cdot 8H_2O$	491.3	501.8	522.9	546.9	572.2	591.7	Geipel et al. (2000)
UO_2(O-phospho-L-threonine)	483.7	501.8	523.4	546.8	572.6	601.0	Günther (2006)
UO_2(fructose-6-phosphate)	478.9	497.1	519.0	543.3	568.9	598.0	Koban et al. (2004)
U(VI)/*Lupine*, shoot		502.4	522.8	549.1	568.7		Günther et al. (2003)
U(VI)/*B. sphaericus*		501.9	523.9	545.8	571.1		Panak et al. (2000)

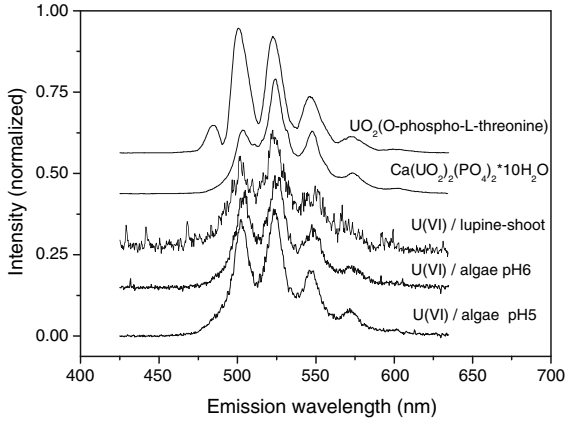

Fig. 5 Comparison of U(VI) luminescence spectra of different biological samples and model compounds

The current study display TRLFS as a powerful tool for the characterization of uranyl complexes formed with biomass, covering also changes of the speciation in the initial and the final supernatant of the sorption experiment. The results proved that phosphate groups are the most important functional groups for uranium binding on algal cells in the middle pH range. The results are comparable with those obtained for the interaction of U(VI) with higher plants and different strains of bacteria.

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