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**Calorimetrically determined U(VI) toxicity in *Brassica napus*  
correlates with oxidoreductase activity and U(VI) speciation**

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**1 ABSTRACT**

2 Radioecological studies depend on the quantitative toxicity assessment of environmental  
3 radionuclides. At low dose exposure, the life span of affected organisms is barely shortened  
4 enabling the transfer of radionuclides through an almost intact food chain. Lethality-based  
5 toxicity estimates are not adequate in this regime because they require higher  
6 concentrations. However, increased radionuclide concentration alters its speciation,  
7 rendering the extrapolation to the low dose exposure chemically inconsistent. Here, we  
8 demonstrate that microcalorimetry provides a sensitive real-time monitor of toxicity of  
9 uranium (in the U(VI) oxidation state) in a plant cell model of *Brassica napus*. We introduce  
10 the calorimetric descriptor “metabolic capacity” and show that it correlates with  
11 enzymatically determined cell viability. It is independent of physiological models and robust  
12 against the naturally occurring fluctuations in the metabolic response to U(VI) of plant cell  
13 cultures. In combination with time-resolved laser-induced fluorescence spectroscopy and  
14 thermodynamic modeling, we show that the plant cell metabolism is affected predominantly  
15 by hydroxo-species of U(VI) with an IC<sub>50</sub> threshold of ~90 μM. The data emphasize the yet  
16 little exploited potential of microcalorimetry for the speciation-sensitive ecotoxicology of  
17 radionuclides.

18

**19 Keywords**

20 Uranium, plant cells, metabolism, isothermal microcalorimetry, speciation, TRLFS,  
21 thermodynamic modeling

22

## 23 INTRODUCTION

24 The transfer of environmental radionuclides into the food chain is a central concern in the  
25 safety assessment of both nuclear waste repositories and remediation strategies in  
26 radioactively contaminated areas. The interaction of radionuclides with plants is mostly  
27 described by transfer factors without knowledge of underlying mechanisms. However,  
28 recent studies of the interaction of radionuclides, e.g., uranium, with plants revealed the  
29 importance of radionuclide speciation. The latter was correlated with uranium uptake from  
30 nutrient medium and translocation in plants.<sup>1</sup> Cross-species studies showed that both  
31 processes are speciation-dependent<sup>2,3</sup> and uranium toxicity can be further modulated by  
32 phosphate.<sup>4</sup> In addition to speciation effects on uranium uptake and oxidative stress  
33 response,<sup>5,6</sup> the redox state of uranium and the intracellular glutathione pool have also  
34 been investigated in plants.<sup>7</sup> The *in situ* speciation of uranium in plants<sup>8</sup> and their subcellular  
35 compartments<sup>9</sup> has been observed by spectroscopy. In response to heavy metal stress,  
36 plants synthesize protective metal-binding metabolites, store metal chelates in the vacuole  
37 or secrete them into the rhizosphere.<sup>10</sup> This reduces metal toxicity which originates in the  
38 replacement of natural metal cofactors from enzymes, the functional inhibition of sulfhydryl  
39 group-containing proteins or the accelerated formation of reactive oxygen species.<sup>10</sup>

40 Correlating molecular information on radionuclide speciation and biomolecular  
41 interactions with physiological performance is a major challenge for quantitative  
42 radioecology. We have recently shown that microcalorimetric monitoring of metabolic  
43 activity in combination with genetic engineering can identify molecular details of the  
44 modulation of uranium toxicity in a living microorganism.<sup>11</sup> However, the application of  
45 microcalorimetry to plant metabolism poses unique challenges. In contrast to bacteria which  
46 exhibit exponential growth phases from which division rates can be derived as

47 physiologically meaningful parameters of toxicity, this is not the case with plant cells. Their  
48 metabolism does not follow simple mathematical models and typically declines under the  
49 conditions of calorimetric measurements, where photosynthesis is not supported. Whereas  
50 non-photosynthetic experimental conditions are compatible with plant cell culture, there is a  
51 lack of model-free descriptors that can be used to derive quantitative measures of toxicity  
52 from metabolic monitoring. Such descriptors would greatly enhance the value of  
53 microcalorimetry in radioecology, because metabolic monitoring has reached a degree of  
54 sensitivity that allows detecting actinide toxicity in the environmentally relevant  
55 concentrations. Here, lethality is negligible and thus inadequate to derive realistic toxicity  
56 measures. In contrast, metabolic responses are clearly visible and render themselves the  
57 most sensitive and also biologically most meaningful sensor of toxicity.

58 In order to overcome these restrictions, we have empirically determined a model-  
59 independent descriptor, i.e., “metabolic capacity”, that allows evaluating calorimetric data  
60 of declining metabolic phases as typically found with plant cells. In the present work, we  
61 used this approach to investigate the concentration-dependent influence of U(VI) on plant  
62 cell metabolism using canola callus cells (*Brassica napus*). *B. napus* is known to be able to  
63 accumulate heavy metals in higher quantities than many other species.<sup>3</sup> The callus cells are  
64 superior over non-callus cells due to their simpler organization and the better control of  
65 their growth conditions. At the same time, they retain the ability to synthesize typical  
66 secondary metabolites of intact tissues,<sup>12</sup> which renders them a suitable model system for  
67 metabolic studies. The “metabolic capacity” of the cells in the presence of 0 to 200  $\mu\text{M}$  U(VI)  
68 was determined and correlated with cell viability. We were particularly interested in relating  
69 these data to uranium speciation. Therefore, the latter was further assessed by time-  
70 resolved laser-induced fluorescence spectroscopy and thermodynamic modeling. Our data

71 provide both a physiological validation of the descriptor “metabolic capacity” and a  
72 speciation-dependent quantification of U(VI) toxicity in a well-established plant cell model.

73

#### 74 **MATERIALS AND METHODS**

75 **Cell Cultivation.** Callus cells from *B. napus* (PC-1113) were obtained from DSMZ  
76 (Braunschweig, Germany). Friable cells were transferred to liquid modified Linsmaier and  
77 Skoog medium (medium R<sup>13</sup>) to initiate growth of suspension cell cultures on an orbital  
78 shaker at room temperature. After a 7 days growth cycle the cells were subcultured into  
79 fresh culture medium to maintain the suspension culture. Cell cultures from passage number  
80 2 – 11 were used for microcalorimetry and viability measurements.

81 **Microcalorimetry.** Cell suspensions (20 mL) were filtered through a nylon mesh (50 µm  
82 pore size) without suction. Subsequently, the cells were rinsed with 10 mL medium R with a  
83 reduced phosphate concentration of 12.5 µM representing 1% of the original phosphate  
84 concentration (medium R<sub>red</sub>; pH 5.8; Table S1, supporting information) in order to minimize  
85 precipitation of U(VI) phosphate complexes in experiments with uranium. Isothermal  
86 calorimetric measurements were performed with a TAM III (Thermal Activity Monitor)  
87 instrument (Waters GmbH, Eschborn, Germany) equipped with 12 microcalorimeters in twin  
88 configuration (one side for the sample the other for an aluminium reference). Sample  
89 preparation time was kept as short as possible. Wet cells (0.3 g) were transferred into 4 mL  
90 ampoules with 2 mL of medium R<sub>red</sub>. A U(VI) stock solution was prepared by dissolution of  
91 UO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub> × 6 H<sub>2</sub>O in Milli-Q water (Milli-RO/Milli-Q-System, Millipore, Molsheim, France)  
92 and subsequent filtration through 0.2 µm filters (Filtropur S, Sarstedt, Nümbrecht,  
93 Germany). The final concentration was 9.58 mM UO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub> as determined by inductively  
94 coupled plasma-mass spectrometry (ICP-MS; model ELAN 9000, Perkin Elmer, Boston, USA).

95 Aliquots of this stock solution were added to the cell suspensions giving final concentrations  
96 of 20-200  $\mu\text{M}$  U(VI). The ampoules were tightly capped, including control samples of  
97 medium  $R_{\text{red}}$  and of cells in medium  $R_{\text{red}}$  in the absence of U(VI). The samples were held in  
98 the TAM III in a waiting position for 15 min before complete insertion followed by 45 min  
99 equilibration. In each experiment, thermograms were recorded at least in duplicates in the  
100 absence and in the presence of different U(VI) concentrations. Seven independent  
101 microcalorimetric experiments with different cell passages were evaluated (details are given  
102 in Fig. 2). Over the course of the experiments (up to 300 hours), the pH stayed between 5.5  
103 and 5.9.

104 **Viability Measurements.** Cell viability was measured by the MTT test.<sup>14</sup> It detects the  
105 activity of mitochondrial and cytosolic dehydrogenases which reduce water-soluble 3-(4,5-  
106 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Duchefa, Harlem, The  
107 Netherlands) to the water-insoluble formazan product accompanied by a yellow to blue  
108 color change.<sup>15</sup> A stock solution of MTT (5 mg/mL) was prepared in phosphate buffered  
109 saline (PBS, without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; Biochrom, Berlin, Germany) and passed through a 0.2  $\mu\text{m}$   
110 filter (Filtropur S). At the end of calorimetric data acquisition, the pH values of the nutrient  
111 media were measured within the ampoules (inoLab pH meter pH720 and SenTix Mic pH  
112 electrode, WTW, Weilheim, Germany). Subsequently, the supernatant medium was removed  
113 and the cells were washed twice with 1 mL PBS solution followed by the addition of 1 mL  
114 PBS and 200  $\mu\text{L}$  MTT stock solution to each sample. The ampoules were sealed and  
115 incubated in the dark under gentle agitation for 3 hours at room temperature. The  
116 supernatants were removed and 1 mL 0.04 M HCl (p.a., Merck Darmstadt, Germany) in  
117 isopropanol (p.a., Roth, Karlsruhe, Germany) was added to each cell sample to dissolve the  
118 formazan crystals. After slight agitation for 10 min at room temperature,  $8 \times 100 \mu\text{L}$  of each



119 isopropanol solution was pipetted into 96-well plates (CELLSTAR®, Greiner Bio-one,  
120 Frickenhausen, Germany) and the absorbance at 620 nm determined in a microplate reader  
121 (Mithras LB940, Berthold, Bad Wildbad, Germany). Viability of U(VI)-exposed cells was  
122 expressed as the absorbance in percent of that from non-exposed control samples. The  
123 results represent mean values and standard errors of the mean of a total of 12-24  
124 independent samples.

125 **Time-Resolved Laser-Induced Fluorescence Measurements.** Time-resolved laser-induced  
126 fluorescence (TRLFS) measurements were performed under ambient conditions at room  
127 temperature in order to characterize the U(VI) speciation in medium R<sub>red</sub>. Aliquots of the  
128 9.58 mM UO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub> stock solution were added to medium R<sub>red</sub> to give final concentrations of  
129 20-200 μM U(VI). The pH values of these solutions were readjusted to pH 5.8 with diluted  
130 NaOH (Merck) solutions, if necessary. TRLFS studies were performed using a Nd:YAG  
131 pumped OPO system (New-Port Spectra Physics, Quanta Ray, USA) with a repetition rate of  
132 20 Hz and laser energies of about 4.4 mJ.<sup>9</sup> The excitation wavelength was 440 nm. Emission  
133 signals were focused on the entrance slit of a 270 mm spectrograph (SP2300, Acton  
134 Research, Roper Scientific, Martinsried, Germany) and the luminescence spectra detected  
135 with an intensified camera system (PIMAX3, Princeton Instruments, Roper Scientific). Using  
136 the internal delay generator, time-resolved spectra were recorded during a gate width of  
137 500 ns. In 101 delay steps the gate was shifted to delay times of 0.5 or 5 μs with a step size  
138 of 5 or 50 ns, respectively. At each delay time, spectra were recorded with 100 laser pulses  
139 per spectrum in the wavelength range between 450.4 and 727.0 nm at a resolution of  
140 0.266 nm. The start wavelength of about 450 nm was selected to avoid scattered light from  
141 the exciting laser pulse on the camera. The emission spectra were recorded with WinSpec32

142 (Roper Scientific), converted into ASCII files and evaluated with the OriginPro 2015G  
 143 software (OriginLab Corporation, Northampton, USA).

144

## 145 RESULTS AND DISCUSSION

146 **Isothermal Microcalorimetry.** We have used heterotrophically growing *B. napus* cells as a  
 147 model system to investigate interference of U(VI) with plant metabolism. Figure 1 (A and B)  
 148 shows an example of two unprocessed thermograms obtained from two independent cell  
 149 cultures in the presence of three different concentrations of U(VI) nitrate.

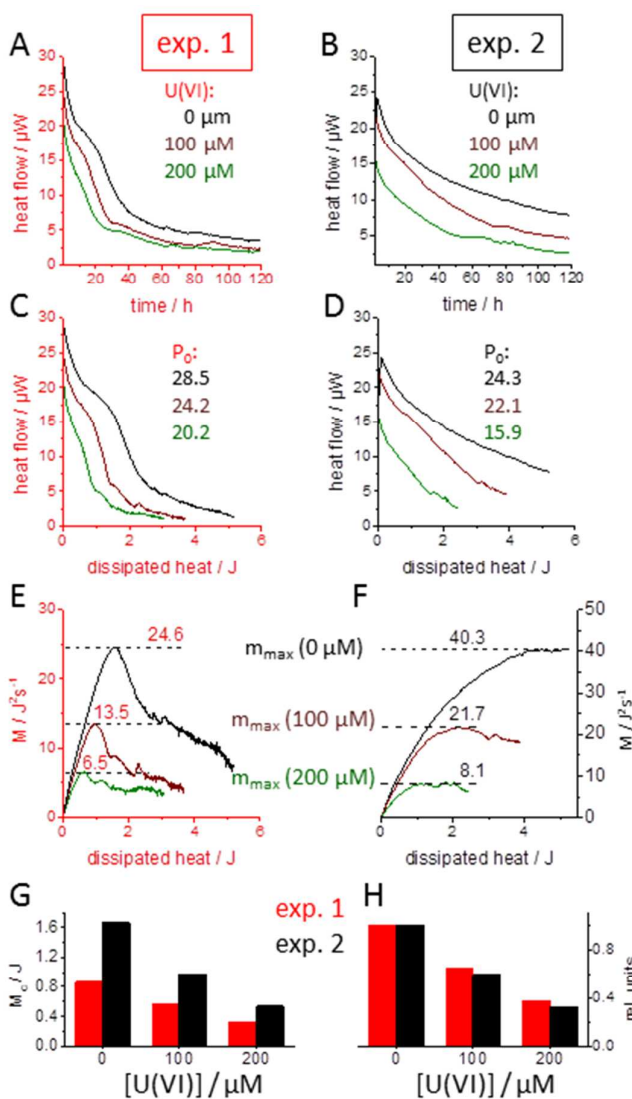


Figure 1: U(VI) dependence of the “metabolic capacity” for distinctly different temporal declines of metabolic activity. Thermograms from two independent *B. napus* cell cultures (left and right panels in A to F) were recorded at 0, 100 and 200  $\mu\text{M}$  U(VI) (raw data in A and B), transformed into enthalpy plots (C and D) and the “metabolic capacity”  $M$  calculated (in  $\text{J}^2\text{s}^{-1}$ , E and F). G) The “characteristic metabolic capacity”  $M_c$  (in J) was obtained by dividing the  $M_{\text{max}}$  values by the initial thermal power  $P_0$  of each thermogram (indicated in C and D). H) For comparison, the  $M_c$  values from panel G were scaled to unity with respect to the values obtained in the absence of U(VI).

152 All traces show a temporal decrease in metabolic thermal power and a general reduction  
153 of heat production with increasing U(VI) concentration. Despite these robust general trends,  
154 the shape of the metabolic decline was biphasic in one case (Fig. 1 A) and resembled an  
155 exponential decay in the other (Fig. 1 B). Variability in the thermograms corresponding to  
156 cells from different passages was generally observed but typically less than in the depicted  
157 examples, where the standard deviation between thermograms (scaled to unity at time  
158 zero) was 27%. Consequently, there is no obvious time point at which the comparison of  
159 heat flow data would result in an unambiguous ranking of metabolic activity in dependence  
160 of U(VI) concentration. Since enzyme activity and substrate supply are the salient  
161 determinants of metabolic activity, we have searched for a quantitative descriptor related to  
162 these two parameters in order to compare metabolic states.

163 Whereas enzyme activity generates thermal power ( $\mu\text{W}$ ), the overall substrate depletion  
164 is related to the total dissipated heat (J), because the latter is produced from substrate  
165 consumption. We define the “metabolic capacity”  $M$  as the product of the time-dependent  
166 heat flow  $P$  (overall “enzyme activity”) and the integrated dissipated heat  $H$  (overall  
167 “substrate depletion”) relative to a time zero ( $t_0$ ):

$$168 \quad M(t) := P(t) \cdot H(t), \quad \text{with} \quad H(t) = \int_{t_0}^t P(t) dt. \quad (1)$$

169  $M(H)$  can be calculated from an “enthalpy plot”, i.e., the plot of  $\frac{dH}{dt}$  ( $= P$ ) as a function of  
170  $H$  (Fig. 1 C and D), by multiplication of each original data pair. The result is graphically  
171 displayed for the two independent experiments in Figs. 1 E and F. Since  $H(t_0)$  and  $P(t^\infty)$  are  
172 zero, there will always be a characteristic time  $t_c$  and enthalpy  $H_c = H(t_c)$  at which  $M(H)$   
173 reaches a maximum  $M_{\max}$  in units of  $\text{J}^2\text{s}^{-1}$ . Despite the differently shaped original  
174 thermograms, the decrease of  $M_{\max}$  with increasing U(VI) toxicity was surprisingly similar for  
175 the two experiments. Finally, the  $M_{\max}$  values were normalized with respect to the initial

176 heat flow  $P_0 (= P(t_0))$  of each trace (Fig. 1 C and D), resulting in the “characteristic metabolic  
177 capacity”  $M_c$  which carries the unit Joule (Fig. 1 G). The virtually identical U(VI) sensitivity of  
178 the cells in the two experiments is best appreciated when the  $M_c$  values are further scaled to  
179 those measured in the absence of uranium (Fig. 1 H). Whereas the raw data show the  
180 metabolic activity at a given time point, the derivation of  $M_c$  contains the full history of the  
181 thermogram up to the time  $t_c$  at which  $M_{max}$  was reached. The integrative evaluation of  
182 thermograms according to Eq. 1 markedly reduced the variation in the  $M_c$  values between  
183 the two experiments as compared to their original  $P(t)$  values at any given time and U(VI)  
184 concentration. Importantly,  $M_c$  is unambiguously defined and thus independent of  
185 subjective choices of data points or physiological models for quantitating toxicity on the  
186 basis of metabolic activity. The high reproducibility of the U(VI) sensitivity expressed by  $M_c$   
187 values derived from the differently shaped thermograms was surprising. In order to address  
188 the physiological relevance of the purely phenomenologically derived value  $M_c$ , we analyzed  
189 the calorimetric data from seven independent cell cultures (from which 37 thermograms  
190 were evaluated) and asked whether the  $M_c$  values correlated with representative enzyme  
191 activities which are typically used as markers for cell viability and are routinely measured by  
192 an MTT test of oxidoreductase activity.<sup>14</sup> The latter was performed either directly after the  
193 calorimetric recordings, i. e., at low residual metabolic activity after  $M_c$  had been surpassed,  
194 or in cell cultures outside the calorimeter under otherwise identical conditions. Calorimetric  
195 and enzymatic data were averaged among the studied cultures.

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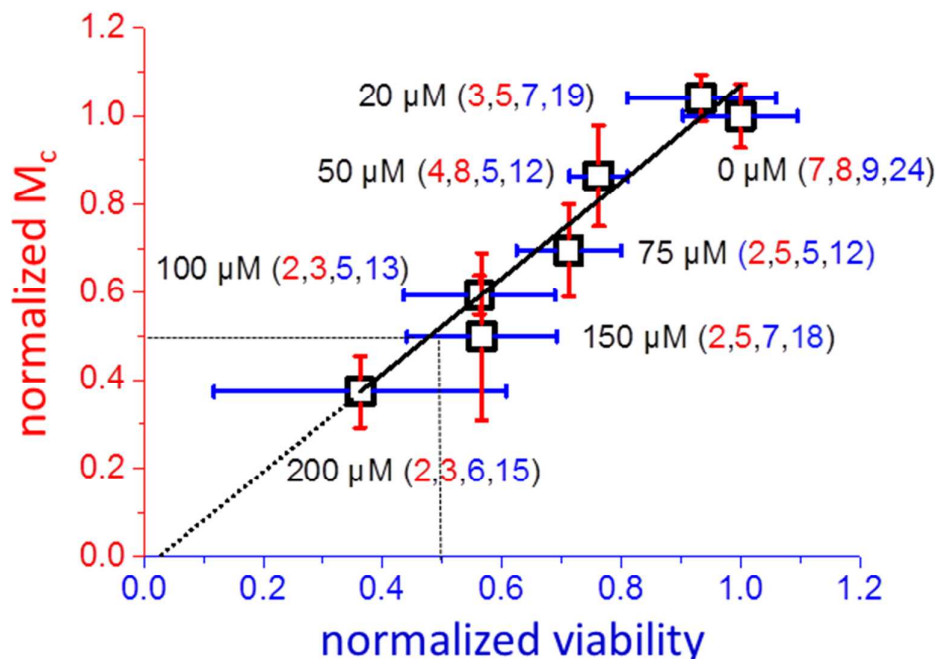


Figure 2: Correlation of oxidoreductase activity (viability) with “characteristic metabolic capacity”  $M_c$ . Data were averaged from experiments carried out with several cell cultures (Fig S3). The first and second number in brackets indicates how many cell cultures and thermograms contributed to each microcalorimetric data point, respectively. The third and fourth number give the corresponding information on cell cultures and individual MTT tests. For both assays, the data were normalized to the respective average of the values obtained with control samples in the absence of U(VI). Solid line: linear regression (slope of 1.09 / standard deviation 0.087; intercept -0.02 / standard deviation 0.065; Pearson’s  $R = 0.98$ ). The average standard deviation in the calorimetric (red error bars) and oxidoreductase data (blue error bars) is 15.4 % and 21.9 %, respectively. Dotted lines: projection of half maximal inhibition on the regression line corresponds to an  $IC_{50}$  of  $\sim 160 \mu\text{M}$  U(VI).

200 Figure 2 shows that oxidoreductase activity and  $M_c$  are to a very good approximation  
 201 linearly related at U(VI) concentrations up to 200  $\mu\text{M}$ . The evaluation clearly supports the  
 202 physiological relevance of the definition of  $M_c$ . Therefore, we suggest the use of the  
 203 “characteristic metabolic capacity”  $M_c$  as a calorimetric descriptor of metabolic activities  
 204 under conditions, where metabolism does not follow simple growth models but declines  
 205 continuously and with varying time dependence. In the case of *B. napus* cell culture, the  $M_c$

206 values reveal a half-maximal inhibitory concentration of 160  $\mu\text{M}$  U(VI) which agrees with the  
207 MTT viability test due to the almost ideal correlation. The variance in the calorimetric data is  
208 smaller and derived from integration over data interval of typically hours, as compared to  
209 the MTT test which captures oxidoreductase activity at a subjectively chosen time point.  
210 However, the data set is still too small to claim statistical significance (e.g. by a Students t-  
211 test).

212 **Calculation of the U(VI) Speciation.** The effects of U(VI) on the cell metabolism may  
213 depend on its speciation in the medium, i.e., its complexation with biomolecules, organic  
214 and inorganic anions. The speciation is known to change with concentration, culture medium  
215 composition, and pH value. Therefore, it was further analyzed under the conditions of the  
216 calorimetric experiments (supporting information). Table 1 summarizes the main U(VI)  
217 species in the medium  $R_{\text{red}}$  at pH 5.8 at different U(VI) concentrations (sample 1-6). Their pH  
218 dependence is shown in Fig. S1.

219 Under the experimental conditions, the solid  $\text{UO}_2\text{HPO}_4$  phase dominates at 20  $\mu\text{M}$   $\text{U(VI)}_{\text{tot}}$   
220 followed by a significant amount of  $(\text{UO}_2)_3(\text{OH})_5^+$ . The latter increases with  $\text{U(VI)}_{\text{tot}}$  and is the  
221 dominating species already at  $\text{U(VI)}_{\text{tot}} > 50 \mu\text{M}$ . Besides that,  $(\text{UO}_2)_4(\text{OH})_7^+$ ,  $\text{UO}_2\text{OH}^+$ ,  $\text{UO}_2^{2+}$ ,  
222 and  $(\text{UO}_2)_2(\text{CO}_3)(\text{OH})_3^-$  species are formed, whereas the absolute amount of  $\text{UO}_2\text{HPO}_4$  (s)  
223 remains constant due to the limited phosphate concentration in medium  $R_{\text{red}}$ .

224 The calculations included only a single solid U(VI) phase. The significant formation of other  
225 phases, i.e., metaschoepite or becquerelite, would be possible, however, can be neglected  
226 under the applied conditions. All U(VI) solutions with medium  $R_{\text{red}}$  were treated by  
227 ultracentrifugation (1 hours, 280,000  $\times g$ ). Due to the ultracentrifugation, a decrease of the  
228 U(VI) concentrations in the solutions was observed (see Table S2 supporting information),  
229 which can be attributed to the sedimentation of  $\text{UO}_2\text{HPO}_4(\text{s})$ . This decrease is higher than

230 presumed assuming only the formation of  $\text{UO}_2\text{HPO}_4(\text{s})$ , however, lower than expected for  
 231 the additional formation of significant amounts of metaschoepite and becquerelite (see  
 232 Supporting Information). Thus, we assign the insoluble fraction of U(VI) mainly to  
 233  $\text{UO}_2\text{HPO}_4(\text{s})$  and only small amounts of secondary mineral phases and / or U(VI) in colloids  
 234 formed with nutrient components.

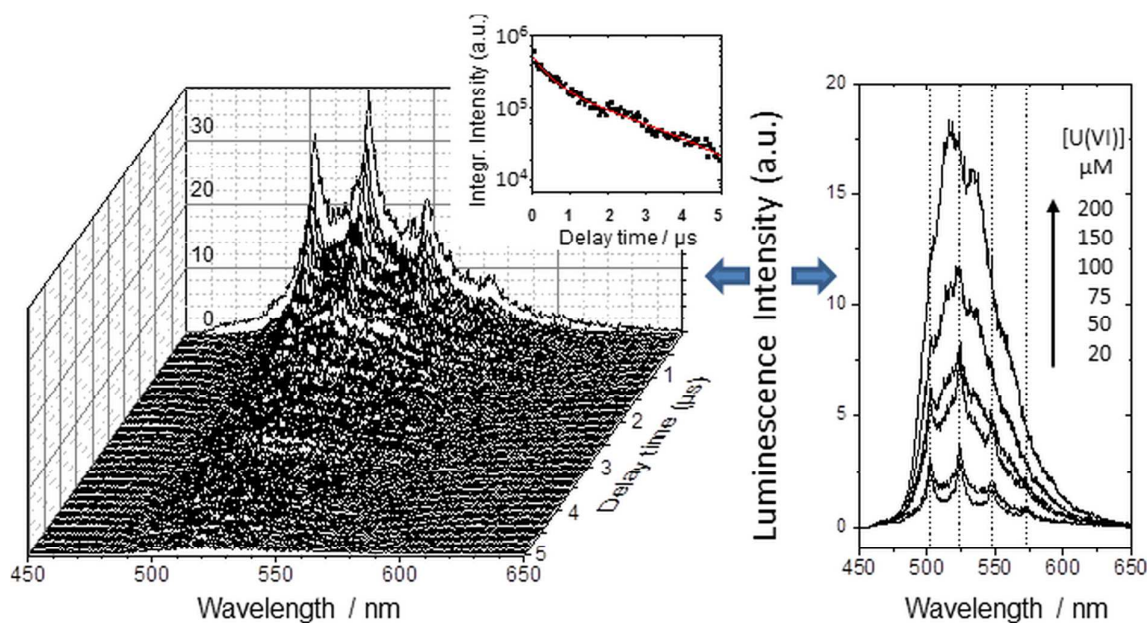
235 **Table 1:** Calculated U(VI) speciation in medium  $R_{\text{red}}$  with solid phases restricted to  $\text{UO}_2\text{HPO}_4$   
 236 (s) only (pH = 5.8,  $p\text{CO}_2 = 10^{-3.5}$  atm). Species that do not exceed 4% of the total U(VI)  
 237 concentration ( $\text{U(VI)}_{\text{tot}}$ ) at any of the analyzed  $\text{U(VI)}_{\text{tot}}$  values are not shown (see Supporting  
 238 Information for further details).

Sample	$\text{U(VI)}_{\text{tot}}$ (mol/L)	$(\text{UO}_2)_2(\text{CO}_3)^-$ $(\text{OH})_3^-$	$(\text{UO}_2)_3(\text{OH})_5^+$	$(\text{UO}_2)_4(\text{OH})_7^+$	$\text{UO}_2\text{OH}^+$	$\text{UO}_2^{2+}$	$\text{UO}_2\text{HPO}_4(\text{s})$
1	$2.0 \times 10^{-5}$	3.66	17.20	1.41	6.10	4.34	62.50
2	$5.0 \times 10^{-5}$	5.42	49.00	7.70	4.70	3.34	25.00
3	$7.5 \times 10^{-5}$	5.21	56.80	10.71	3.76	2.68	16.67
4	$1.0 \times 10^{-4}$	4.94	60.50	12.80	3.18	2.26	12.50
5	$1.5 \times 10^{-4}$	4.48	63.87	15.80	2.47	1.75	8.33
6	$2.0 \times 10^{-4}$	4.13	65.50	17.90	2.05	1.46	6.25

239

240

241 **Experimental Analysis of U(VI) Speciation.** TRIFS measurements were carried out in  
 242 medium  $R_{\text{red}}$ . Figure 3(A) shows the time-resolved luminescence spectrum at 50  $\mu\text{M}$  U(VI)  
 243 and the corresponding decay curve. The latter required bi-exponential approximation,  
 244 indicating the presence of at least two luminescent species.



245

Figure 3: Time-resolved laser fluorescence spectroscopy. A) Spectral dependence of the time-resolved decay of the luminescence of 50  $\mu\text{M}$  U(VI) in medium  $R_{\text{red}}$  ( $\text{pH} = 5.8$ ) and the decay curve of the integral luminescence intensity (inset). B) Luminescence spectra of U(VI) in medium  $R_{\text{red}}$  as a function of the total U(VI) concentration (delay time: 51 ns). The vertical lines indicate the peak positions of the initial phosphate species.

246 Due to the presence of high amounts of organic substances as well as  $\text{Fe}^{3+}$  and  $\text{Cl}^-$  (cf.  
 247 Table S1, supporting information), dynamic quenching processes decreased the  
 248 luminescence lifetimes, thereby, hampering comparisons with model compounds.  
 249 Therefore, only the spectral shapes but not the lifetimes were further analyzed. Figure 3 (B)  
 250 shows U(VI) luminescence spectra recorded at a delay time of 51 ns at different  
 251 concentrations of  $\text{U(VI)}_{\text{tot}}$ . With increasing  $\text{U(VI)}_{\text{tot}}$ , the spectra changed significantly  
 252 indicating a change of the predominant U(VI) solution species. To attribute the spectra to  
 253 U(VI) species, spectra at different delay times were analyzed by peak deconvolution using  
 254 the peak fitting module of OriginPro 2015G and compared to literature data (Table S3,  
 255 supporting information).



256 The concentration-dependent U(VI) spectra measured after 51 ns (Fig. 3B) lead to specific  
257 peak maxima obtained after spectral peak deconvolution. Whereas the peak maxima in  
258 samples 1, 5, and 6 stayed constant with increasing delay times, the peaks in samples 2 and  
259 3 showed a progressive hypsochromic shift over time, indicating the predominance of  
260 different U(VI) species at different delay times (Figure S2, supporting information). The  
261 occurrence of at least two luminescent solution U(VI) species was already concluded from  
262 the rough lifetime estimation. The comparison of the peak maxima with those of reference  
263 species (Table S3, supporting information) shows that U(VI) phosphate species dominated in  
264 sample 1 and contributed also to the luminescence of sample 2-4 at 51 ns. At longer delay  
265 times, the spectra corresponded to  $(\text{UO}_2)_3(\text{OH})_5^+$  which was also the case for sample 5 and 6.  
266 For the latter, the constancy of the spectra over all delay times demonstrated the  
267 dominance of this species at  $\text{U(VI)}_{\text{tot}} > 100 \mu\text{M}$ . Although the luminescence lifetimes of all  
268 samples were generally strongly quenched, a shorter lifetime of the U(VI) phosphate species  
269 was observed than for the U(VI) hydroxo species (not shown). This agrees with literature  
270 data, where luminescence lifetimes of  $6.0 \mu\text{s}$  and  $4.7 \pm 0.1 \mu\text{s}$  were reported for  $\text{UO}_2\text{HPO}_4$   
271 and  $(\text{UO}_2)_x(\text{PO}_4)_y$ , respectively<sup>16</sup> in contrast to  $19.8 \pm 1.8 \mu\text{s}$  for  $(\text{UO}_2)_3(\text{OH})_5^+$ .<sup>17</sup> The TRIFS  
272 results are further supported by the thermodynamic speciation calculations:  $\text{UO}_2\text{HPO}_4$  and  
273  $(\text{UO}_2)_3(\text{OH})_5^+$  dominates the U(VI) speciation at  $20 \mu\text{M}$  and  $\geq 50 \mu\text{M}$   $\text{U(VI)}_{\text{tot}}$ , respectively (cf.  
274 Table 1).

275 **U(VI) Speciation and Metabolic Activity.** Microcalorimetry has been used previously for  
276 the assessment of U(VI)-dependent bacterial activities both in genetically engineered  
277 bacteria<sup>11</sup> or communities found in uranium waste heaps.<sup>18</sup> Typically, calorimetric studies  
278 have been evaluated based on mathematically well-defined growth models<sup>19</sup> or enthalpy  
279 balances<sup>20</sup> in order to derive per cell estimates of metabolic rates. However, metabolic

280 calorimetry suffers from the lack of quantitative descriptors when simple growth models  
281 cannot be applied. This is particularly the case with plant cells in the presence of heavy  
282 metals which exhibit metabolic decline rather than growth. On the other hand, calorimetry is  
283 particularly attractive for assessing radionuclide toxicity in the environmentally relevant low  
284 dose exposure. Calorimetry is non-invasive, toxicity estimates can be derived directly from  
285 the physical response of a living system, and experimental procedures enable minimal  
286 handling efforts with radionuclides. In order to exploit the potential of microcalorimetry in  
287 radioecological investigations, we have established a generally applicable evaluation of  
288 thermograms from cells that exhibit metabolic decline without a preceding growth phase.  
289 We have shown here that the “characteristic metabolic capacity”  $M_c$  (Eq. 1) is a reliable  
290 calorimetric descriptor that scales with enzymatically determined viability in *B. napus* cells.  
291 Using this descriptor, the influence of U(VI) speciation on metabolic activity can be  
292 addressed. The TRLFS data and the thermodynamic modeling showed that the total amount  
293 of U(VI) hydroxo complexes in fresh medium  $R_{red}$  increased with  $U(VI)_{tot}$ , whereas the low  
294 phosphate concentration in medium  $R_{red}$  limited  $UO_2HPO_4(s)$  formation to less than 13  $\mu M$ .  
295 Figure 4 correlates  $M_c$  with  $U(VI)_{tot}$  and, alternatively, with the sum of the two hydroxo  
296 complexes that make up more than 50% of all species for  $[U(VI)_{tot}] > 20 \mu M$  (Table 1). The  
297 normalized  $M_c$  values scale linearly with the U(VI) hydroxo complexes. Importantly, the data  
298 intersect the y-axis nearly perfectly at the expected value of  $M_c = 1$  for [U(VI) hydroxo  
299 complexes] = 0. Although  $M_c$  varies linearly also with  $[U(VI)_{tot}] > 0$ , the required condition of  
300  $M_c = 1$  for  $[U(VI)_{tot}] = 0$  is not met. The data indicate that the dissolved hydroxo-species  
301 possess highest bioavailability. In contrast, species that are not bioavailable ( $UO_2HPO_4(s)$ )  
302 contribute to  $U(VI)_{tot}$  without lowering  $M_c$ . This explains the right-shift of the plot of  $M_c$  vs.  
303  $U(VI)_{tot}$ . Correspondingly, metabolism is suppressed by 50% at a concentration of  $\sim 90 \mu M$  of

304 the U(VI) hydroxo-complexes (intersection of regression line with x-axis, Fig. 4) which is  
 305 significantly less than the ca. 160  $\mu\text{M}$  estimated from the regression line in Fig. 2, where the  
 306 indicated  $[\text{U(VI)}]_{\text{tot}}$  concentration includes also non-bioavailable U(VI) species.

307 Speciation-dependence of U(VI) uptake and translocation is a general phenomenon. Both  
 308 are affected by pH in *Arabidopsis thaliana* plants, although pH also altered the cellular redox  
 309 balance in this organism.<sup>5</sup> In contrast to the majority of invasive biochemical studies on U(VI)  
 310 uptake and toxicity, isothermal microcalorimetry can provide a highly sensitive and non-  
 311 invasive monitor of radionuclide-mediated metabolic effects. We have shown here for *B.*  
 312 *napus* cells that metabolic responses of plant cells to U(VI) can be measured under the non-

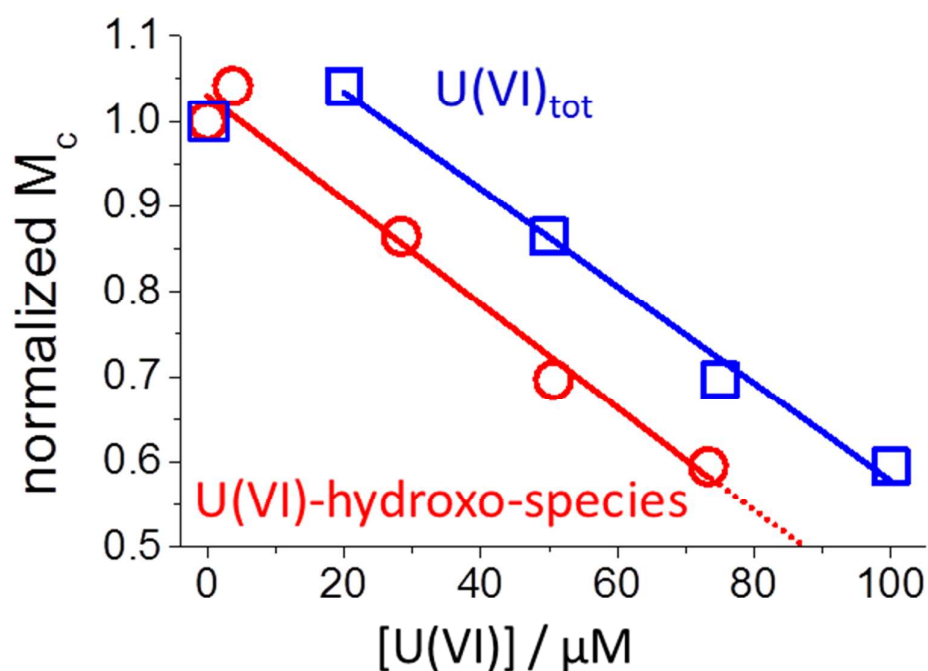


Figure 4: U(VI) species-dependency of the “characteristic metabolic capacity”  $M_c$ . Circles: the normalized  $M_c$  values (from Fig. 2) are plotted vs. the concentration of the dominant hydroxo-species, i.e.,  $[(\text{UO}_2)_3(\text{OH})_5^+] + [(\text{UO}_2)_4(\text{OH})_7^+]$ . Squares: the same  $M_c$  values plotted vs.  $\text{U(VI)}_{\text{tot}}$ . Solid lines: linear regression of the data. The strong correlation (Pearson's  $R = 0.99$ ) of  $M_c$  with the U(VI)-hydroxo-species (red) indicates that it is biologically the most relevant (dotted line: extrapolation to  $\text{IC}_{50}$  of  $\sim 90 \mu\text{M}$ ).

313 photosynthetic regime of microcalorimetry. Remarkably, the “characteristic metabolic  
314 capacity”  $M_c$  correlates sufficiently well with an established enzymatic viability test to be  
315 used as a quantitative descriptor of toxicity. It appears less variable than the enzymatic test,  
316 which is probably due to the integration of thermal data over time as opposed to the single  
317 time point estimates obtained by the MTT test. The current observation is statistically not  
318 significant (by Student’s t-test) but a systematic comparison of error margins in  $M_c$   
319 determinations and enzymatic assays will be interesting once larger data sets are available.  
320 The current study demonstrates the potential of life cell microcalorimetry for radioecological  
321 studies, enabling viability measurements independently of prior genetic or detailed  
322 physiologic analyses. At the same time, handling of radionuclides can be reduced to an  
323 absolute minimum. Although microcalorimetry has been shown to also reveal mechanistic  
324 details on uranium toxicity when linked to genetic engineering<sup>11</sup>, such data complement but  
325 cannot replace studies on uranium-dependent specific enzyme activities (for example  
326 Saenen et al. 2015). Instead, the strength of life cell microcalorimetry originates in its quick  
327 and non-invasive systemic approach. It appears particularly suited for the future  
328 quantification of toxicity mediated by internal exposure to other  $\alpha$ - and  $\beta$ -emitting uranium  
329 isotopes for which the present work provides the necessary reference. It remains to be  
330 elucidated to which extent such investigations can contribute to the ecotoxicological risk  
331 assessment of radionuclides, where endpoints at higher degrees of biological complexity  
332 such as metabolism-driven processes (growth and development) may provide the missing  
333 link to the effects of radionuclides at the molecular scale<sup>21</sup>.

334

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338 measurements, and S. Weiss for ultracentrifugation.

339

340 Associated Content

341 **SUPPORTING INFORMATION**342 **Thermodynamic Modeling of the U(VI) Speciation in Medium R<sub>red</sub>**

343 Table S1, S2, Fig. S1

344 **Analysis of TRLFS Data**

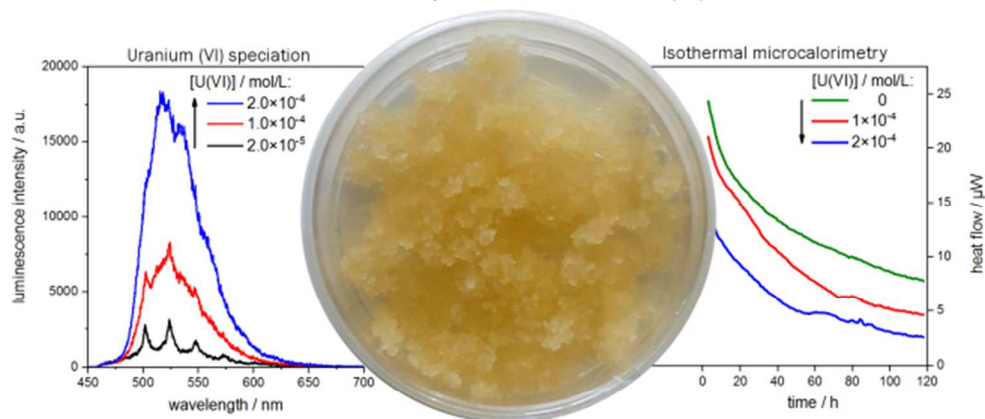
345 Table S3, Fig S2

346 **Dose response curves of metabolic capacity and oxidoreductase activity**

347 Fig. S3

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- 399

*Brassica napus* cells + uranium(VI)

TOC graphical abstract

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