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► **To cite this version:**

Benjamin Reeves, Maria Rosa Beccia, Pier Lorenzo Solari, Danil Smiles, David Shuh, et al.. Uranium Uptake in *Paracentrotus lividus* Sea Urchin, Accumulation and Speciation. *Environmental Science and Technology*, 2019, 53 (14), pp.7974-7983. 10.1021/acs.est.8b06380 . hal-02196756

HAL Id: hal-02196756

<https://hal.science/hal-02196756v1>

Submitted on 12 Feb 2020

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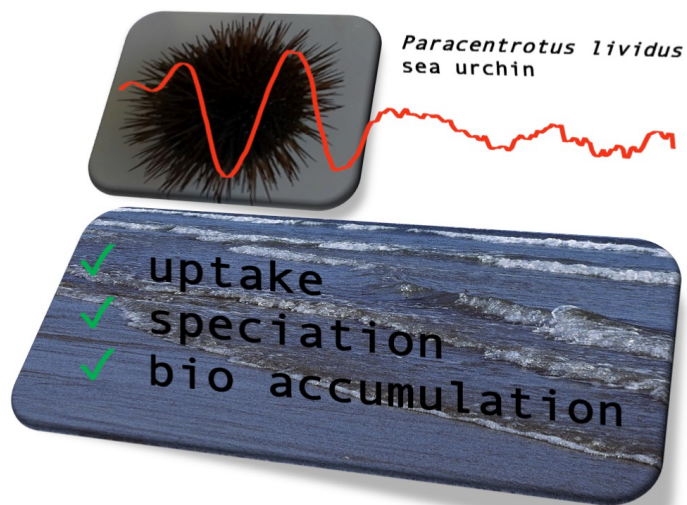
Journal:	<i>Environmental Science & Technology</i>
Manuscript ID	es-2018-06380c.R1
Manuscript Type:	Article
Date Submitted by the Author:	10-May-2019
Complete List of Authors:	Reeves, Benjamin; Universite Cote d'Azur, ICN Beccia, Maria Rosa; Universite Cote d'Azur, Institut de Chimie de Nice Solari, Pier Lorenzo; Synchrotron Soleil, Smiles, Danil; Chemical Sciences Division, Glenn T. Seaborg Center Shuh, David; Chemical Sciences Division, Glenn T. Seaborg Center Berthomieu, Catherine; CEA-Cadarache, Laboratoire des Interactions Protéine Métal Marcellin, Didier; CEA-Cadarache, Laboratoire des Interactions Protéine Métal Bremond, Nicolas; CEA, DRF/BIAM Passeron Mangialajo, Luisa; COMUE Sorbonne Universites; Universite Cote d'Azur, Ecomers Pagnotta, Sophie; Universite Cote d'Azur, CCMA Monfort, Marguerite; CEA DAM Ile de France Moulin, Christophe; CEA DAM Ile de France Den Auwer, Christophe; Universite Cote d'Azur, ICN

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Uranium uptake in *Paracentrotus lividus* sea urchin, accumulation and speciation

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Abstract

Uranium speciation and bioaccumulation were investigated in the sea urchin *Paracentrotus lividus*. Through accumulation experiments in a well-controlled aquarium followed by ICP-OES analysis, the quantification of uranium in the different compartments of the sea urchin was performed. Uranium is mainly distributed in the test (skeletal components), as it is the major constituent of the sea urchin, but in terms of quantity of uranium per gram of compartment, the following rating: intestinal tract > gonads >> test, was obtained. Combining both extended X-ray Absorption Spectroscopy (XAS) and time resolved laser induced fluorescence (TRLFS) spectroscopic analysis, it was possible to identify two different forms of uranium in the sea urchin, one in the test, as a carbonato-calcium complex, and the second one in the gonads and intestinal tract, as a protein complex. Toposome is a major calcium-binding transferrin-like protein contained within the sea urchin. EXAFS data fitting of both contaminated organs *in vivo* and the uranium-toposome complex from protein purified out of the gonads revealed that it is suspected to complex uranium

43 in gonads and intestinal tract. This hypothesis is also supported by the results from two imaging techniques
44 *i.e.* Transmission Electron Microscopy (TEM) and Scanning Transmission X-ray Microscopy (STXM).
45 This thorough investigation of uranium uptake in sea urchin is one of the few attempts to assess the
46 speciation in a living marine organism *in vivo*.

47
48

49 INTRODUCTION

50 Uranium is a natural radioelement present in the earth's crust under its natural isotope distribution (^{Nat}U :
51 $^{238}U = 99.275\%$, $^{235}U = 0.719\%$ and $^{234}U = 0.0057\%$). It is a very weak radiotoxin (^{Nat}U specific activity =
52 25767 Bq/g) but most importantly, a chemical toxin, as it is able to interact with various biological targets
53 resulting in heavy metal poisoning. Its crustal concentration ranges between 0.3 and 12 mg/kg, depending
54 on the geological composition. Due to anthropogenic activities, uranium is also present in the environment
55 as technologically enhanced naturally occurring radioactive materials (TENORM) where mining activities
56 are or have been implemented, mostly for nuclear fuel applications. Additional anthropogenic origins of
57 uranium in the environment may also result from nuclear power accidents and nuclear weapons activities.¹⁻
58 ³ Last, in some particular areas of military conflict, the use of depleted uranium in munition components has
59 resulted in the dispersion of uranium metal into the environment.⁴ Most importantly, because uranium is a
60 limited issue of public health to date (except in some specific mining or contaminated zones as mentioned
61 above), it serves as a model (uranium is easy to manipulate in the laboratory) for more radioactive actinyls
62 of the early actinide family, *i.e.*, neptunyl and plutonyl, predominantly in pentavalent oxidation state (+V)
63 or, in specific oxidative conditions, as hexavalent (+VI).

64 In most environmental and biological conditions, uranium mainly occurs in its hexavalent oxidation
65 state, in the form of the di-oxo uranyl cation $\{UO_2^{2+}\}$. Uranyl, if bioavailable, may compete with essential
66 biological metal cations in binding proteins, affecting all the biological processes that depend on them.⁵ For
67 example, the coordination mechanisms of uranyl with the iron binding protein transferrin has been explored
68 several times, by Pible *et al.* in 2006,⁶ by Vidaud *et al.*⁷ in 2007, by Hemadi *et al.* in 2009,⁸ and more
69 recently by theoretical approaches by Wang *et al.*⁹ It was shown that the uranyl ion can compete with iron,
70 which could potentially lead to the internalization of uranium in the cytoplasm of cells. However, the
71 bioavailability and potential transfer of uranyl strongly depends on its physico-chemical speciation. For
72 instance, several studies showed that uranyl bioavailability decreases when it is bound to some inorganic
73 ligands (e.g., phosphate, carbonate) or adsorbed on colloidal and particulate matter.¹⁰ This is why it is
74 essential to deeply understand its speciation in the biosphere and biocycles, to evaluate the health risk
75 engendered on living organisms and potentially humans, through the trophic chain.

76

77 Seawater comprises the largest percentage of the hydrosphere (*ca.* 96.5 %) and covers about 71% of the
78 earth's surface.¹¹ It is also the final environmental repository for contaminated waters from rivers and basins.
79 In oceans and seas, uranium is naturally occurring at an average concentration of around 10^{-8} M although,
80 as for the earth's crust, heterogeneities apply.¹² In 1956, Rona *et al.* reported a concentration of uranium
81 between 3.1 and 3.5 $\mu\text{g}/\text{kg}$ in sea water at different locations, *i.e.*, in the North Atlantic, the Gulf of Mexico,
82 and in the Straits of Florida.¹³ Ku *et al.* reported similar values with a mean concentration of uranium of
83 about 3.3 $\mu\text{g}/\text{L}$.¹⁴ Altogether uranium represents about 1% of the total radioactivity in seawater (the major
84 contributor being ^{40}K accounting for more than 90%).¹⁵ In seawater, the accumulation of several heavy
85 metals in marine organisms has been widely studied at all trophic levels.¹⁶⁻¹⁸ Indeed, a wide diversity of
86 organisms has been investigated, from simple organisms like algae to more complex ones like fish. It is far
87 beyond the scope of this introduction to make an exhaustive report on this topic. Concerning radionuclides
88 specifically, the IAEA (International Atomic Energy Agency) has continuously updated Concentration
89 Factor (CF) values that could be used for impact calculations.¹⁹⁻²⁰ The CF is defined as the ratio between
90 the concentration of the element of interest in the studied organism and the concentration of the element in
91 the surrounding medium. The IAEA reported values of CF for ^{137}Cs and ^{90}Sr in different species of the biota,
92 from algae to fish, and evaluated the distribution inside the organism, in multiple locations in the Baltic
93 Sea.¹⁹ Jeffree *et al.* recently studied the accumulation and the speciation of ^{241}Am , ^{109}Cd , ^{57}Co , ^{51}Cr , ^{134}Cs ,
94 ^{54}Mn and ^{65}Zn in spotted dog fish and turbot.²¹ The distribution inside the organism was determined, and
95 even though similarities were observed between some of the elements, the distribution is still element-
96 dependent. Recently, Maloubier *et al.* studied the bioaccumulation of ^{241}Am and ^{152}Eu in the marine sponge
97 *Aplysina Cavernicola* and reported speciation data for europium.²² Some recent work has also focused only
98 on uranium. Barillet *et al.* showed that uranium is highly bioaccumulated in Zebrafish *Danio Rerio*, and
99 also that it can affect some of the biological functions, like hepatic defences.²³ Eb-Levadoux *et al.* also
100 showed that in Zebrafish, uranium is reprotoxic due to a potential interaction with proteins.²⁴ However, to
101 the best of our knowledge, there is no other data on uranium speciation inside marine organisms, probably
102 because of the very low concentration of this element in seawater (ppb levels), which challenges the use of
103 spectroscopic methods for speciation assessment. Nonetheless, speciation data obtained *in vivo* are essential
104 to shift from a large-scale descriptive approach and inventories to a well-informed biochemical mechanistic
105 approach.

106
107 In previous work, we investigated the uranium speciation in seawater, showing that in these conditions,
108 it is mainly present as a dicalcium uranyl tricarbonate complex, $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$.²⁵ This form of uranyl has
109 already been reported in aqueous natural systems.²⁶ It has also been shown not to be bioavailable when it
110 occurs in natural drinking waters.²⁷ In the present report, we are addressing the question of uranium

111 speciation upon bioaccumulation in sea urchin *Paracentrotus lividus* (Figure 1) by proceeding to *in vivo*
112 contamination experiments in a simplified and model biotope. We chose *P. lividus* because it is widely
113 distributed throughout the Mediterranean Sea and the north-eastern Atlantic. Moreover, *P. lividus* is often
114 used as a biochemical indicator of local pollution because of its sedentary habits and well-known sensitivity
115 to pollutants. It is known to accumulate heavy metals like zinc, lead, copper, iron or cadmium.²⁸⁻²⁹ For
116 instance, Warnau *et al.* reported the concentration of several heavy metals (Zn, Pb, Cd, Fe, Cu, Cr and Ti)
117 in the sea grass *Posidonia oceanica* and in the sea urchin *Paracentrotus lividus*, from three different
118 locations in the Mediterranean Sea.³⁰ They also measured the quantity of each studied metal in the three
119 different compartments of the sea urchins; the test (skeleton = shell + spines), the intestinal tract, and the
120 gonads. They reported that the metal ion accumulation changes with the body compartment. For most metal
121 ions, accumulation is ranked in the following order: digestive tube and gonads \geq test. This is also what was
122 observed one year later with other elements, *i.e.*, Ag, Cs and Am.³¹ Our objective here was to assess the
123 speciation of uranium in the different compartments of *P. lividus* in order to decipher the accumulation
124 biomechanisms. To do so, we first described the bio-distribution of uranium in the aquarium and within the
125 sea urchin. We have then assessed the uranium speciation in the main organs of the sea urchin (test, gonads
126 and intestinal tract) with two spectroscopic X-ray probes that are complementary, namely X-ray Absorption
127 Spectroscopy (XAS under both XANES and EXAFS regimes) and Scanning Transmission X-ray
128 Microscopy (STXM) elemental imaging. They have been combined with Time Resolved Laser
129 Fluorescence Spectroscopy (TRLFS) data and Transmission Electron Microscopy (TEM) images. In a last
130 step, the toposome protein, which is the main protein present in the sea urchin organs, was extracted and
131 purified out of the gonads.³² As the toposome is a transferrin like protein and is acting as a Ca reservoir for
132 sea urchins, it should be considered as a potential candidate for uranyl binding in the gonads. The speciation
133 of uranyl in a solution containing the purified toposome was investigated.

134

135 **EXPERIMENTAL SECTION**

136 **Seawater, sea urchin collection and aquarium setup**

137 Seawater was collected in the Mediterranean Sea at the Environmental Laboratory of the International
138 Atomic Energy Agency (IAEA) at 30 m from the coast of Monaco, 50 m deep (43° 43' 49" N, 7° 25' 40").
139 The seawater was filtered at 0.2 μm (Whatman, GF/C grade) and sterilized by UV treatment to eliminate
140 particles and microorganisms. Commercial silica gravel (850 g) was placed at the bottom of the aquarium
141 filled with 10 L of seawater. The silica gel was needed to ensure the survivability of the sea urchins inside
142 the aquarium, and played no chemical role, nor interfered significantly with the experiments. Only one sea
143 urchin was placed in the aquarium at the same time. The aquarium was equipped with a filter and an air

144 pump that were turned on 7 days before placing the sea urchins inside to equilibrate the whole system. The
145 seawater temperature was maintained at 16°C using a water-cooling system during the experiments.

146 *Paracentrotus lividus* sea urchins (Figure 1) were collected in Villefranche-sur-mer, by the Laboratoire
147 Océanographique de Villefranche (UMR 7093, Mediterranean Sea, France) and were fed with native algae
148 until 3 days before contamination. The food was then removed from the aquarium, and the water was cleaned
149 of any remnants before the first uranium spike. Specimens with similar size were chosen (average diameter
150 = 7-8 cm, average total dry mass = 20 g). All results reported in this work concern experiments performed
151 on female specimens. Each specimen used in this report is described in Table S1 of Supplementary
152 Information (SI) file.

153

154 **Spiking procedure and uranium distribution**

155 Uranium nitrate $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ was directly dissolved in diluted nitric acid (0.1 M) to obtain the 0.375 M
156 uranium solution spike for aquarium use. Both nitric acid and uranium nitrate were of reagent grade, and
157 deionized water was used to dilute the nitric acid. Every 24 hours, 500 μL of this solution were introduced
158 in the aquarium, to reach a theoretical final concentration of $[\text{U}] = 1.88 \cdot 10^{-4}$ M after 10 days. Prior to the
159 spiking (around 10 min before), 500 μL of a $2 \cdot 10^{-4}$ M solution of sodium hydroxide were introduced in the
160 aquarium to avoid any modification of the pH. The latter was controlled using commercial pH paper
161 designed for seawater. The measured pH was around 8. The uranium concentration corresponds to a total
162 mass of uranium of 476.20 ± 27.12 mg. It was chosen as the best compromise between uranium natural
163 concentration and EXAFS sensitivity.

164 Each sample from the aquarium (sea urchin or gravel) was rinsed with deionized water before any further
165 analysis, to remove any uranium potentially adsorbed on the sample surface, and of remnant contaminated
166 sea water, to ensure the validity of the results. The uranium content of each sample was analyzed by ICP-
167 OES (details are provided in SI file).

168

169 **Toposome extraction and purification.**

170 The toposome purification was performed according to Castellano *et al.* with slight modifications (details
171 are provided in SI file).³³ The final concentration was estimated via UV-visible, using a calculated epsilon,
172 to be around 35 mg/mL ($\epsilon = 1.252$), corresponding to a molar concentration in monomer units of about $2 \cdot 10^{-4}$
173 M. The epsilon was calculated using the sequence of amino acids published by Noll *et al.*³⁴ The toposome
174 was then frozen until further use. The protein purity is estimated to be 80% minimum. The toposome is
175 probably organized in the form of trimers. The two discernible fractions under the main band probably
176 correspond to the presence of the two isoforms described at 200 kDa and 180 kDa for the *Paracentrotus*
177 *lividus* toposome, which are present in the nutritive phagocytes of the gonads.³⁵

178

179 Time Resolved Laser Induced Fluorescence spectroscopy (TRLFS)

180 Sample preparation, sea urchin: The test was dried, then crushed into powder. The powder was directly
181 analyzed with no further preparation. Concerning the gonads, they were dried and also directly analyzed
182 with no further preparation.

183 Sample preparation, toposome-uranium complex: The same procedure as described above was used.
184 However, the uranium was this time dissolved in a solution of Tris/HCl (10mM) NaCl (10mM), pH 5.5, to
185 reduce the ionic strength of the solution (Cl concentration). The solution was then kept at 4°C until analysis.

186 Data acquisition: A Nd-YAG laser (Model Surelite Quantel) operating at 355 nm (tripled) and delivering
187 about 10 mJ of energy in a 10 ns pulse with a repetition rate of 10 Hz, was used as the excitation source.
188 The laser output energy was monitored by a laser power meter (Scientech). The focused output beam was
189 directed onto the urchin part (gonad, shell) of the sea urchin (previously crushed) placed in a 1 mm
190 pathlength quartz cell of the spectrofluorometer (F900 Edinburgh). The detection was performed by an
191 intensified charge coupled device (Andor Technology) cooled by Peltier effect (-5°C) and positioned at the
192 polychromator exit for the emission spectra measurement and by a photomultiplier tube (PMT) to measure
193 fluorescence decay time. Logic circuits, synchronized with the laser shot beam, allowed the intensifier to be
194 activated with determined time delay (from 0.005 to 1000 μ s) and during a determined aperture time (from
195 0.005 to 1000 μ s). From a spectroscopic point of view, various gate delays and durations were used to ensure
196 the presence of only one complex by the measurement of a single fluorescence lifetime and spectrum.
197 Fluorescence lifetime measurements were performed by varying the temporal delay with fixed gate width.

198

199 X-ray Absorption Spectroscopy (XAS) Data Acquisition and Analysis

200 Sample preparation, sea urchins: sea urchins 2, 3 and 5 were analyzed by XAS: gonads and intestinal
201 tract for sea urchin 2 (EXAFS), only gonads for sea urchin 3 (EXAFS) and test for sea urchin 5 (X-ray
202 absorption near edge structure (XANES) (see Table S1). For sea urchin 2, gonads and intestinal tract were
203 freeze-dried for 24h. Solid pellets were then prepared by mixing the dry residue with polyethylene in order
204 to obtain homogenous solid pellets. For sea urchin 3, solid pellets were prepared with the gonads by mixing
205 fresh gonads with polyethylene. As polyethylene is only composed of light chemical elements, it does not
206 interfere with the EXAFS measurements. In both cases, the pellets were then kept at -20°C until the analysis
207 to avoid any deterioration of the biological system. For sea urchin 5, the test was mechanically ground and
208 pressed into solid pellets. The Liebigite reference sample ($\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$) was obtained from the
209 mineralogy collection of the Museum National d'Histoire Naturelle (MNHN), Paris, France.

210 Sample preparation, U-toposome complex: uranium nitrate was directly dissolved in Tris/HCl (50mM)
211 NaCl (150mM), pH = 5.5. This pH value prevents any visible precipitation of uranium hydroxides. Absence

212 of hydrolysis was also verified using speciation codes. pH was adjusted to 5.5 with concentrated chlorhydric
213 acid. The solution was then mixed with the protein, to obtain a final concentration of uranium of $8 \cdot 10^{-5}$ M,
214 and a concentration of protein in monomeric units estimated at $1.6 \cdot 10^{-4}$ M. An estimated excess of protein
215 ensures that no free uranyl would remain in the solution, which would interfere with the EXAFS analysis.
216 The solution was then kept at 4°C until analysis.

217 EXAFS data acquisition: experiments were performed on the MARS beamline of the SOLEIL
218 synchrotron facility. Energy calibration was performed at the yttrium K edge at 17038 eV and EXAFS
219 experiments at the U L_{III} edge. The MARS beamline is dedicated to the investigation of radioactive materials
220 in the hard X-ray range.³⁶ The beamline optics consist essentially of a water-cooled double-crystal
221 monochromator (FMB Oxford), which is used to select the incident energy of the X-ray beam and for
222 horizontal focalization, and two large water-cooled reflecting mirrors (IRELEC/SESO) that are used for
223 high-energy rejection (harmonic part) and vertical collimation and focalization. All measurements were
224 achieved in fluorescence mode using a 13-element high purity germanium detector (ORTEC). The X-ray
225 absorption spectra for the test sample (from sea urchin 5) were measured at room temperature, whereas the
226 spectra for the gonad samples (from sea urchins 2 and 3) and for the intestinal tract sample (from sea urchin
227 3) were measured at -165°C . To perform the latter measurements, the samples were inserted in a specifically
228 designed double containment cell (H. Hermange, SOLEIL) and inserted in the dedicated liquid nitrogen
229 cryostat of the beamline. The protein sample was measured at room temperature.

230 EXAFS data processing was performed using the ATHENA code.³⁷⁻³⁸ The E_0 energy was identified at
231 the maximum of the absorption edge. Fourier transform (FT) with k^2 weighting was performed between 2.5
232 and 12 \AA^{-1} for gonads and 10.5 \AA^{-1} for the intestinal tract, with a Hanning window. The fits were performed
233 using the DEMETER code (version Demeter 0.9.25) and were fit in R space between 1 and 5 \AA . EXAFS
234 data fitting: One global amplitude factor S_0^2 and one energy threshold correction factor ΔE_0 were used for
235 every path of the fits. The agreement factor r (%) and the quality factor (QF = reduced χ^2) of the fits were
236 provided directly by DEMETER. Phases and amplitudes were calculated using the FEFF6 simulation code
237 integrated in DEMETER based on a partial structural model (*in silico*) of uranyl-acetate complex
238 ($\text{UO}_2(\text{acetate})_2$.) This model was chosen because it exhibits both monodentate and bidentate carboxylate
239 ligation to the uranyl equatorial plane. The scattering paths used for the fitting procedure are: i) simple
240 scattering paths including U-O_{ax} within the oxo bond, U-O_{eq} corresponding to the equatorial oxygen atoms
241 and U...C corresponding to the C atom of the bidentate carboxylate group; ii) multiple scattering paths
242 including the quadruple path U-O_{ax} within the oxo bond, and the triple scattering U-O-C of the monodentate
243 carboxylate function. During the fitting procedure, the number of atoms of carbon in the monodentate and
244 bidentate functions was let free, in case only one coordination mode was present. The total number of
245 variables in the fit was equal to 12.

246
247 **Transmission electron microscope (TEM) imaging**
248 Gonads and intestinal tract of sea urchin 2 were analysed with TEM. Directly after dissection, fresh gonads
249 and fresh intestinal tract samples were fixed for 2 h at room temperature with 2.5% glutaraldehyde in
250 cacodylate buffer (0.1 M, pH 7.4) in artificial seawater, then washed with 0.1 M cacodylate buffer (pH 7.4)
251 and post-fixed with 1 % osmium tetroxide in cacodylate buffer containing 1% potassium ferrocyanide. The
252 samples were embedded in Epon resin after dehydration using an acetone/water solution and then acetone.
253 Ultrathin sections (70–80 nm) were cut using a diamond diatom mounted on an ultramicrotome (Ultracut S,
254 Leica) and placed on copper TEM grids coated with formvar film. Sections were observed with a JEOL
255 JEM 1400 TEM equipped with a CCD camera (Morada, Olympus SIS) at the Centre for Applied Microscopy
256 (CCMA, University of Nice Sophia Antipolis, Nice, France).

257
258 **Scanning transmission X-ray microscope (STXM) imaging**
259 Sample preparation: Gonad and intestinal tract cells of sea urchin 2 were analysed with STXM.
260 The STXM samples were prepared as described above for TEM analysis. Sections of 70-80 nm were placed
261 on a 100 nm thick Si₃N₄ membrane window (1 mm square) in a 10 mm frame obtained from Silson Ltd. A
262 second Si₃N₄ window was glued over the first to seal and confine the sample for radiological control
263 purposes.

264 Data acquisition: Data was recorded with the STXM on beamline 11.0.2 of the Advanced Light Source
265 (ALS) located at the Lawrence Berkeley National Laboratory in Berkeley, USA.³⁹ The STXM methodology
266 employed in this study was similar to that described previously.⁴⁰⁻⁴¹ The photon energy calibration of the
267 monochromator was performed at the neon K-edge (867.3 eV). The STXM measurements were performed
268 with a 25 nm zone plate in transmission mode and the ALS was operating in top-off mode with a beam
269 current of 500 mA. Images at a single energy were obtained by raster scanning the sample and collecting
270 X-rays as a function of sample position. Elemental maps of uranium were obtained by subtracting an image
271 taken before the absorption threshold from an image obtained at resonance utilizing the U N₅ transition
272 (~738 eV), following image alignment.

273 Data treatment: Data treatment was performed with the aXis2000 code developed at McMaster
274 University.⁴²

275
276 **RESULTS AND DISCUSSION**
277 **Uranium uptake and distribution in the organs.**
278 We have investigated the uptake of uranium by *P. lividus* in the closed aquarium system described in the
279 experimental section (1 spike per day for 10 days). Four similar experiments have been conducted with five

280 sea urchins that are detailed in Table S1. A control experiment with no spike was also performed at the same
281 time in a similar aquarium next to the one used for contamination. It showed that no detectable uranium was
282 naturally present in sea urchins (below the detection limit of ICP-OES, about 1×10^{-7} mg/L). Each *P. lividus*
283 specimen was sacrificed and dissected at the end of the 10 days. Gonads, digestive tube and test were
284 separated, and uranium content was measured by ICP-OES.

285 First, the total uranium balance was measured in the entire system with sea urchin 4 to ensure that the
286 major part of the uranium was distributed within the main components (sea water, gravel, sea urchin), and
287 not, for instance, adsorbed on the aquarium wall or filter. The concentration of uranium in each of the
288 different system components was measured. The results are presented in Figure S1. From the concentration
289 in seawater, the global Concentration Factor (CF) of sea urchin 4 for an exposure of 10 days was calculated
290 and is 0.37 ± 0.02 .

291 The bioaccumulation of uranium in each compartment of the sea urchin (test, gonad, intestinal tract) has
292 been assessed. Although competition between uranium and other cations was not explicitly taken into
293 account, the use of a natural medium implies that competition is implicitly included. It is presented in Figure
294 2 for sea urchins 1, 2 and 4 as the fraction of total uranium mass per dry weight of each compartment (see
295 Table S2). In the three specimens, the concentration in the digestive tube is almost 3 times and 10 times
296 higher than in the gonads and test, respectively. Differences can be observed between sea urchin 4 and the
297 two others. We explain these discrepancies by the seasonal variations in the gonads quantity and the seasonal
298 variations in the concentration of proteins inside the gonads. Indeed, the experiment on sea urchin 4 was
299 conducted out of the reproduction season (February-May), which was not the case for sea urchins 1 and 2.
300 Partial concentration factors for each compartment may also be calculated. The following CF were obtained
301 for sea urchin 1: $CF_{\text{gonads}} = 1.0 \pm 0.07$, $CF_{\text{intestin}} = 2.8 \pm 0.1$ and $CF_{\text{test}} = 0.25 \pm 0.02$. The overall very low value
302 obtained for the entire specimen is largely due to the low value of CF_{test} that is associated to the largest mass
303 of the specimen (the test). These results are in agreement with the values reported by Warnau *et al.* in 1996
304 as the same ordering was observed for most heavy metals: gonads, intestinal tract \gg test. In their
305 communication, the concentration measured in the intestinal tract is the highest of the three compartments
306 for several heavy metals as iron, copper, tin and mercury. However, the gonads are more concentrated in
307 zinc than the intestinal tract, which means that the distribution is element specific and also confirms that
308 speciation is playing a key role in the accumulation mechanisms. In addition, Warnau *et al.* also highlighted
309 the fact that the concentrations of every metal but lead are always lower in the test than the gonads or the
310 intestinal tract, no matter which of the two latter compartments is the most concentrated. These data clearly
311 highlight the necessity of speciation investigation in each compartment separately, as the accumulation rate
312 of uranium is radically different between the three compartments.

313

314 **Uranium speciation in the test**

315 The test of sea urchin is mainly composed of monocrystalline calcite (calcium carbonate) rich in
316 magnesium.⁴³ Uranium accumulation in test and spines is very low, as mentioned above, with an average
317 concentration of 11 ppm ($CF_{\text{test}} = 0.25 \pm 0.02$). Such a concentration lies just above the estimated EXAFS
318 detection limit under our experimental conditions. Therefore, an EXAFS spectrum could not be recorded
319 from the test with a reasonable signal to noise ratio and only the XANES part of the spectrum was
320 significant. Figure 3 compares the XANES spectra of the test after *in vivo* contamination of sea urchin 5
321 with data acquired from a Liebigite ($\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$) solid state reference. Liebigite is taken here as the
322 model for the main species of uranium in seawater, the dicalcic uranyl tricarbonate species, $\text{Ca}_2\text{UO}_2(\text{CO}_3)_3$.
323 The enlarged insert of Figure 3 shows the derivatives of the spectra. A qualitative comparison of both
324 XANES spectra and their derivatives for the test samples and the Liebigite reference suggests that the
325 speciation of uranium in the test is similar to that in the Liebigite model, although it is not definite proof.
326 This could signify that a sorption mechanism occurs and is at the origin of the uranium accumulation in the
327 test. As adult spines do not grow once they reach their adult size, a mechanism involving sorption on the
328 calcite monocrystalline surface of the spines followed by slow diffusion of uranium inside the calcite
329 structure agrees well with the final very low concentration of uranium in the test, contrary to a mechanism
330 of incorporation of the uranium during the growth of the spines. To complement the XANES results, TRLFS
331 measurements were also performed on sea urchin 5. The wavelengths of the maximum of fluorescence
332 emissions obtained are 471, 488, 507, 528 and 552 nm (Figure 4). Previous studies on uranium compounds²⁵
333 report the wavelengths obtained for different species, including uranium in seawater, several carbonated
334 calcium-uranium complexes, and sulphate and phosphate uranium complexes. Indeed, a slight
335 hypsochromic shift is noticed here, mainly for the higher wavelengths compared to free uranyl (reported
336 wavelengths: 470–488–509–534–559 nm), which is characteristic of $\text{M}_x\text{-UO}_2\text{-(CO}_3)_y$ complexes (with $x :$
337 $1\text{-}2$ and $y : 2\text{-}3$), with $\text{M} = \text{Ca}^{2+}, \text{Mg}^{2+}$ and Sr^{2+} .
338 Even though it is not possible to differentiate all the possible species, the results above are consistent with
339 the presence of the dicalcic uranyl tricarbonate species in the test at a relatively low level.

340

341 **Uranium accumulation in the gonad and intestinal tract cells, imaging and spectroscopy**

342 Transmission electron microscope (TEM) imaging was performed on the gonad cells of contaminated sea
343 urchin 2. Figure 5 shows a large field TEM image of the gonads. More specifically, two types of cells can
344 be observed; the reproductive cells (circular and darker), and the storage cells (various shapes, lighter). No
345 evidence of uranium precipitates can be noted on this micrograph in any of the cells although uranium is
346 present in the gonads at a concentration of around 50 ppm, as mentioned before. Precipitates of uranium
347 phosphate phases have been observed for different bacterial systems. For instance, Suzuki *et al.* reported

348 that nanoprecipitates of uranium were visible extra-cellularly in *Deinococcus radiodurans* after exposure.⁴⁴
349 Uranium-phosphate crystals were also reported to be present inside cells of *Stenotrophomonas Maltophilia*
350 by Merroun *et al.*⁴⁵ It was also observed in living cells UMR-106, which are model osteoblastic cells.⁴⁶ The
351 absence of any visible precipitate, although not a definite proof, suggests that uranyl is not incorporated as
352 an insoluble mineral phase. To further investigate this assumption, STXM elemental imaging was performed
353 on gonads cells of the same sea urchin specimen. Figure 6 shows the STXM image recorded at 738 eV, just
354 above the uranium N_V edge. One can clearly distinguish the cell membrane and the different organelles
355 inside. The elemental map was then obtained *via* alignment and subtraction of the STXM image collected
356 at an energy preceding the N_V edge at 725 eV (Figure S5). The elemental map reveals a featureless map
357 with a shadow ring at the location of the cell wall although the contrast in the shadow ring is noticeable but
358 only 2% above background. Thus, the elemental map shows that uranium is not localized in specific hot
359 spots or precipitates to our degree of both spatial and spectral resolution but may be homogeneously
360 distributed around the cell membrane. As a consequence, both TEM and STXM images suggest that uranium
361 may be complexed within the cell by proteins, enzyme or metabolites even though this is clearly not a
362 definitive evidence because only a limited number of cells were observed and the signal of the shadow ring
363 in the uranium elemental map is very weak.

364 Considering the affinity of uranyl for hard donor oxygen groups like in the transferrin binding site
365 (aspartate, tyrosine, carbonate)^{7,47}, complexation of the uranyl cation by carboxylate rich proteins, enzyme
366 or metabolites is a reasonable assumption. This could also explain the low contrast observed in the STXM
367 contrast image because it would be distributed over the entire cell membrane. Such ligation could involve
368 aspartic, glutamic or tyrosine residues, for instance. To further determine the speciation of uranium inside
369 the cells and possible complexation, contaminated gonads of sea urchin 2 and 3 were analysed by EXAFS
370 and XANES spectroscopy at the uranium L_{III} edge. The XANES spectrum of the gonads of sea urchin 2 is
371 provided in Figure 3 and exhibits the expected uranyl pattern. The EXAFS spectra obtained for the gonades
372 of sea urchins 2 and 3 are presented in Figure 7. The experimental spectrum for sea urchin 2 was adjusted
373 with a model composed of 3 scattering shells (O_{yle} , O_{eq} and C) as explained in the experimental section (the
374 spectrum corresponding to sea urchin 3 being superimposable, but at lower signal to noise ratio, is shown
375 but not fitted). The best fit metrical parameters are reported in Table 1, the Fourier transform (modulus and
376 imaginary parts) is provided in Figure S6. The first shell corresponds to the two axial oxygen atoms typical
377 of the uranyl oxocation. The second shell is composed of 5.1(3) O atoms at 2.37(1) Å (average) and the last
378 shell is composed of 3.3(9) C atoms at 2.94(3) Å. Such a coordination sphere is indeed typical of carboxylate
379 rich biomolecules, although a detailed path by path analysis is not possible here in the absence of a specific
380 structural model. Alternative attempts to fit our data with a Liebigite model lead to unrealistic metrical
381 parameters, thus supporting protein or metabolite complexation. Pible *et al.* in 2006 studied the interaction

382 between uranyl and a wide number of proteins.⁶ They calculated and reported the distances between uranium
383 and selected protein structures containing uranyl. Values for the U-O_{eq} distances fall into the range 2.31 -
384 2.61 Å for monodentate carboxylate groups and 2.41 - 2.84 Å for bidentate carboxylate groups. In the
385 specific case of transferrin, Hémadi *et al.* in 2009 and Wang *et al.* more recently reported the binding
386 mechanism of uranyl to transferrin by DFT calculations.⁸⁻⁹ In the model of Wang, the most probable
387 coordination sphere is composed of one bidentate carbonate with U-O at 2.46 Å, two tyrosines with U-O at
388 2.30 Å, and one monodentate aspartate at a distance of 2.44 Å. The values we obtained for the U-O_{eq} average
389 distance (reported in Table 1) seems to indicate the presence of monodentate carboxylate groups although
390 only average values are discussed here. Concerning the carbons atoms, the U...C average distances reported
391 in Table 1 in the range 2.9 - 3.0 Å also support the presence of bidentate carboxylate functions. No evidence
392 of monodentate complexation was found using the U...C interaction. However, this is not an absolute proof,
393 considering that this interaction is often very weak, due to the angle of the U-O-C bond (deviating from
394 focusing effect optimum angle, 180°) in monodentate configuration. On the other hand, the triple scattering
395 path U-C-O was found to be necessary to fit the experimental data. This path being a fingerprint of
396 monodentate ligation, it suggests the occurrence of at least one monodentate carboxylate function. We thus
397 propose here the following mode of coordination: two atoms of oxygen (for the oxo bonds), a mix of
398 monodentate and bidentate carboxylate functions with an unknown ratio, and possibly one water molecule
399 to complement the equatorial coordination sphere. The above structural data therefore suggest that uranyl
400 is coordinated to carboxylate residues of a protein or metabolite.

401 As for the gonad cells, the EXAFS (Figure 7) and XANES (Figure 3) spectra at the L_{III} edge of uranium
402 was recorded for the intestinal tract cells of sea urchin 2. Qualitatively, the spectra are very similar to what
403 is discussed above, suggesting that the same type of complexation occurs in this compartment as well.
404 Experimental and simulated EXAFS spectra are displayed in Figure 7, best fit structural parameters are also
405 reported in Table 1. The U-O_{eq} average distance (2.35(1) Å) is similar to those obtained for the gonad cells
406 (2.37(1) Å) and the same type of U...C path has been used. This suggests that the same biomolecule (or
407 same type) is involved in the uranium complexation in the intestinal tract.

408 In addition, TRFLS measurements were also performed (Figure 4) on the gonads of sea urchin 5. The
409 following fluorescence wavelengths were obtained: 501-521-542 nm. In contrast to what was obtained for
410 the test, a bathochromic effect with respect to free uranyl (488–509–534 nm) is observed, together with two
411 lifetimes of 140 μs and 1600 μs without modification of the spectra. This indicates that the carbonate calcite
412 form of uranyl is not the correct speciation within the gonads, as already supported by the EXAFS data
413 fitting. Bathochromic effects have been reported to occur in the presence of complexing ligands, such as
414 sulphate or phosphonate, the latter leading to the largest shift (main wavelengths: 496-519-545). In the
415 present case, the shift is even more important taken together with the broadening of the spectrum, indicating

416 that most of the uranyl is likely to be complexed with a strong complexing ligand with multiple interaction
417 sites such as macromolecules that establish several local environments (and account for the broadening
418 observed as well as the different lifetimes).

419
420 In conclusion, the best fit EXAFS data support the hypothesis of a complexation of the uranyl with
421 carboxylate rich proteins, enzymes or metabolites in gonads and intestinal tract cells. This assumption is
422 also in full agreement with both STXM and TEM imaging.

423

424 **Uranium-toposome complexation**

425 The toposome is the main protein in sea urchins and is present in both the gonads and intestinal tract. It
426 is described as a multimer of 180 kDa monomers, known as 22S fragment.⁴⁸

427 This protein, also referred to as major yolk protein, was previously identified as a calcium binding iron-
428 less transferrin like protein.³⁵ As a consequence, toposome is a potential candidate for uranyl complexation
429 in the cells. Different final masses are reported, depending on the number of monomers (from 1 to 6). As a
430 consequence, toposome is a potential candidate for uranyl complexation in the cells.

431 Following toposome extraction from gonad cells and purification, the EXAFS spectrum at the L_{III} edge of
432 uranium was recorded for the U-toposome complex in solution. The experimental XANES data, the
433 experimental EXAFS spectra and the best simulated EXAFS spectra are similar to both gonads and intestinal
434 tract spectra although some slight differences appear between 6 and 9 \AA^{-1} (Figure 7). Nonetheless, the
435 similarities between the best fit structural parameters (reported in Table 1) obtained for the three systems
436 strongly suggest that the toposome protein is a good match for uranyl complexation in those two
437 compartments. The slight differences observed in the EXAFS spectra, notably in the wave between 6 and 9
438 \AA^{-1} are not due to differences in the nature of the binding site (like carboxylate *versus* phosphate ligation for
439 instance) but most probably to differences in conformation as indicated by the similarities of the imaginary
440 parts of the moduli of the FT (Figure S6).

441 TRFLS measurements were also performed on a U-toposome solution (2:1 ratio, $[\text{UO}_2] = 1\mu\text{M}$). The
442 spectrum represented in Figure 4 exhibits the same fluorescence wavelengths as observed before (501-521-
443 542nm). However, two other fluorescence wavelengths must be highlighted: 485nm and 465nm, the latter
444 being characteristic of carboxylic functions. This validates the EXAFS interpretation for the U-toposome
445 complex and suggests that the speciation of uranyl in the toposome complex and in gonads and intestinal
446 tract cells is similar. The absence of the 465 nm wavelength in the gonad TRFLS spectrum may be explained
447 because the gonads are not only composed of toposome, even though it is the main protein. Other proteins
448 or metabolites and thus other complexation conformations might be present. The spectrum obtained for
449 gonads is in consequence less specific.

450
451 The fact that the toposome is likely to complex uranyl inside the sea urchin is a result of great interest, as it
452 is a strong evidence of the metabolization of uranyl inside a living organism, from a dicalcic carbonato form
453 in seawater to a protein complex inside the sea urchin. This shows the importance of speciation as a key
454 parameter to understand and evaluate the potential impact on the environment and on humans that this
455 element, and by extension, other similar elements, can have, if released in the environment. There is still an
456 important need to continue these studies, as the speciation of multiple radioisotopes in biocycles remains
457 completely unknown.

458
459 **Associated content**
460 The Supporting Information is available free of charge on the ACS Publications website at ...
461 The SI contains Table S1, which describes the different measurements on the 5 sea urchins. Results dealing
462 about the distribution of the uranium inside the sea urchin organs and inside the aquarium (Table S2 and
463 Figure S1). Elution profiles and SDS gel are presented Figure S2, S3 and S4. Complementary STXM images
464 and EXAFS data are also presented (Figure S5 and S6).

465
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469 The authors declare no competing financial interest.

470
471 **ACKNOWLEDGMENTS:**
472 This work was financed by the DNP (Direction of Nuclear Propulsion) of CEA DAM. We thank the
473 International Atomic Energy Agency, Environmental Laboratory of Monaco for providing the seawater. We
474 also acknowledge the LOV, UMR 7093 for providing the sea urchins and the algae. The XAS experiments
475 were performed at the MARS beamline of the SOLEIL synchrotron facility, Gif sur Yvette, France. This
476 research was supported in part (DES, DKS) by the Director, Office of Science, Office of Basic Energy
477 Sciences, Division of Chemical Sciences, Geosciences, and Biosciences Heavy Elements Chemistry
478 program of the U.S. Department of Energy under Contract Number DE-AC02-05CH11231 at Lawrence
479 Berkeley National Laboratory (LBNL). This research used resources of the Advanced Light Source, which
480 is a U.S. Department of Energy Office of Science User Facility supported under Contract No. DE-AC02-
481 05CH11231 at LBNL. Finally, we thank the Centre Commun de Microscopie Appliquée for the SEM
482 imaging.

483

484 Table 1: Best fit parameters for EXAFS data from the U L_{III} edge. Numbers in brackets are the estimated
 485 uncertainties, numbers in italics have been fixed. σ^2 is the Debye Waller factor of the considered scattering
 486 path. S_0^2 is the global amplitude factor, e_0 is the energy threshold, R_{factor} is the agreement factor of the fit in
 487 % and Q is the quality factor (reduced CHI^2) of the fit.

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Sea urchin 2	First coordination shell	Second coordination shell	Third coordination shell	Fit parameters ^a
<i>Gonads</i>	2 O _{ax} at 1.79 (1) Å, $\sigma^2 = 0.003 \text{ \AA}^2$	5.1 (3) O _{eq} at 2.37 (1) Å, $\sigma^2 = 0.008 \text{ \AA}^2$	3.3 (9) C at 2.94 (3) Å $\sigma^2 = 0.007 \text{ \AA}^2$	$S_0^2 = 1.00$ $e_0 = 1.56 \text{ eV}$ $R_{\text{factor}} = 1.6 \%$ Q = 2
<i>Intestinal tract</i>	2 O _{ax} at 1.79 (1) Å, $\sigma^2 = 0.007 \text{ \AA}^2$	5.8 (8) O _{eq} at 2.34 (1) Å, $\sigma^2 = 0.010 \text{ \AA}^2$	3.8 (7) C at 2.96 (5) Å, $\sigma^2 = 0.001 \text{ \AA}^2$	$S_0^2 = 1.00$ $e_0 = 3.34 \text{ eV}$ $R_{\text{factor}} = 2.2 \%$ Q = 4
<i>Toposome</i>	2 O _{ax} at 1.78 (1) Å, $\sigma^2 = 0.003 \text{ \AA}^2$	5.5 (9) O _{eq} at 2.35 (2) Å, $\sigma^2 = 0.011 \text{ \AA}^2$	3.4 (7) C at 2.89 (3) Å $\sigma^2 = 0.001 \text{ \AA}^2$	$S_0^2 = 1.10$ $e_0 = 3.29 \text{ eV}$ $R_{\text{factor}} = 2.2 \%$ Q = 12

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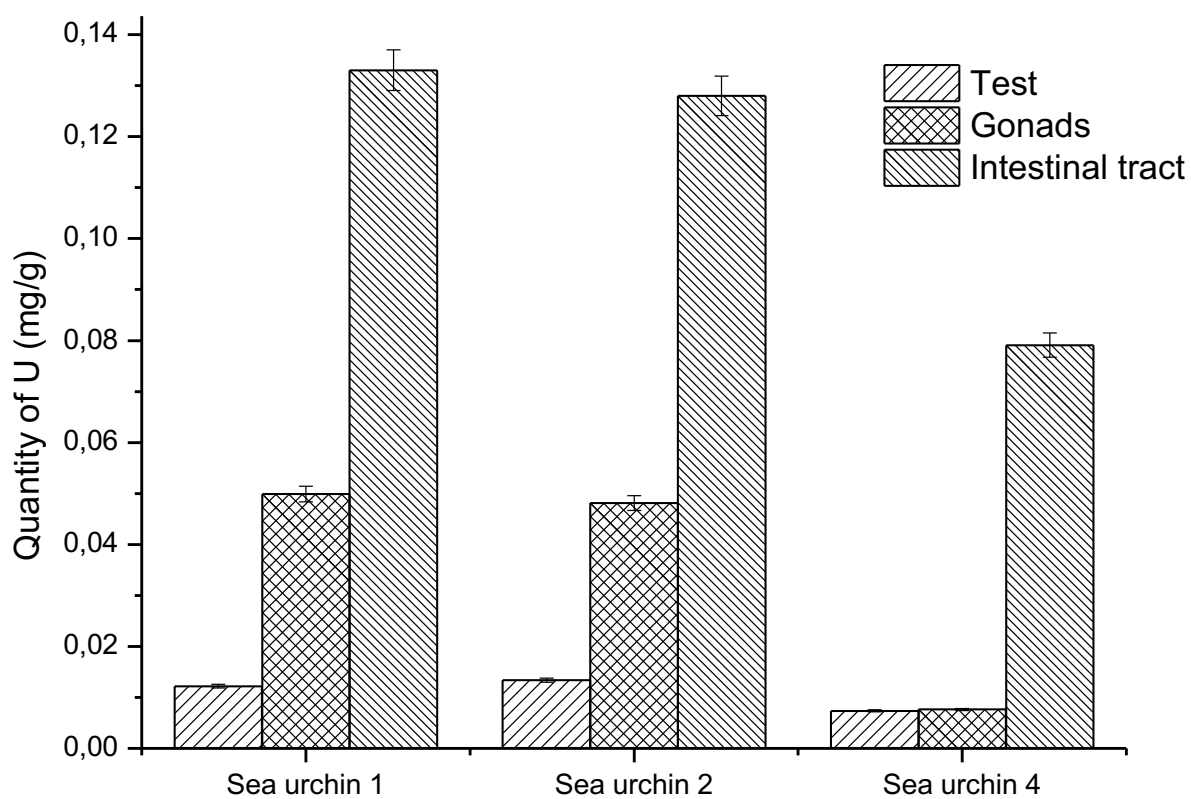
495 Figure 1: Picture of a sea urchin *Paracentrotus lividus* (one unit = one centimeter).

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Figure 2: Uranium concentration (in mg/g, elemental U) for the three compartments of the sea urchins 1, 2 and 4.

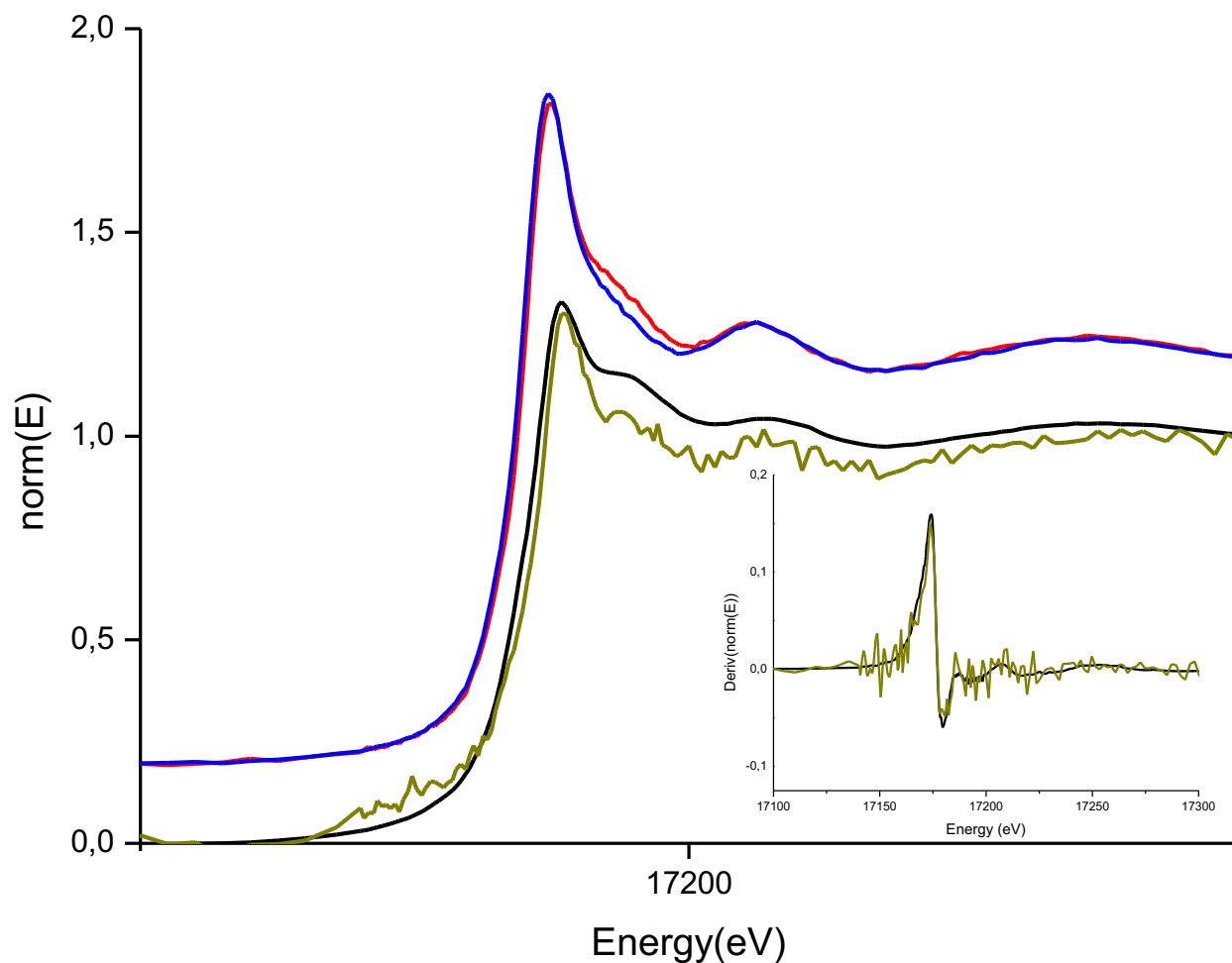
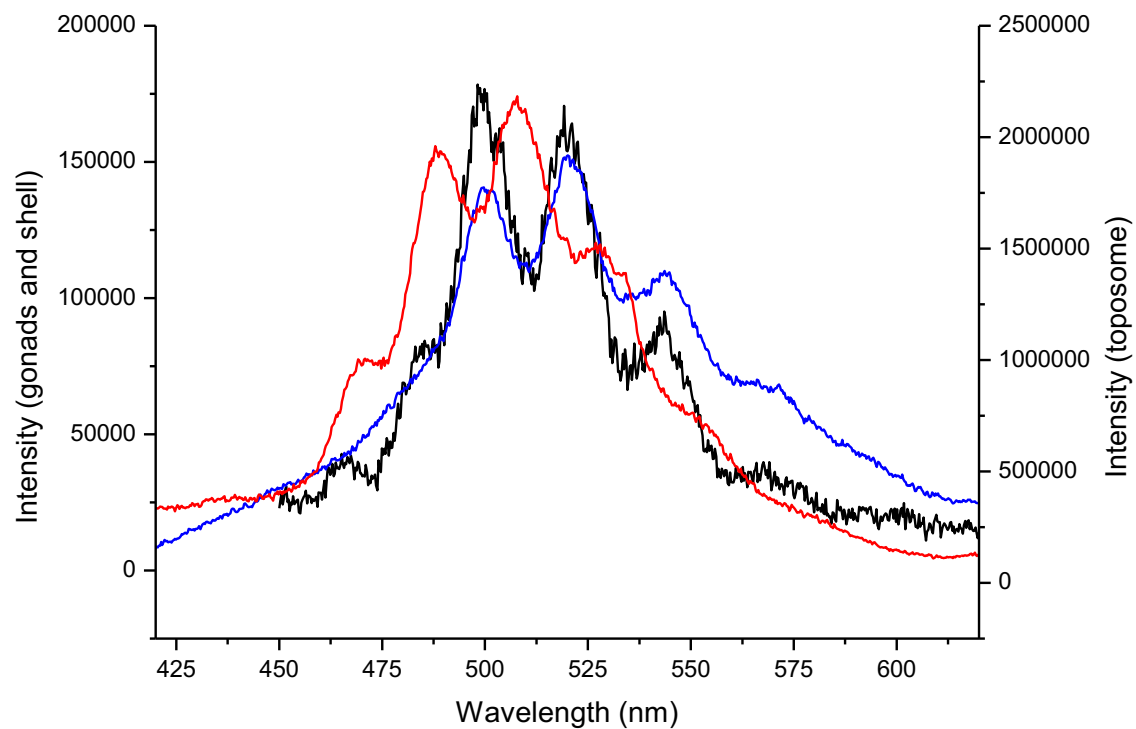


Figure 3: XANES spectra at the U LIII edge of the test of sea urchin 5 (green), of the Liebigite reference sample (black), of the gonads (red) and the intestinal tract (blue) of sea urchin 2 (both shifted in ordinates for clarity). Insert: Enlargement of the corresponding first derivatives of the test and the Liebigite reference sample.

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Figure 4: TRFLS spectra obtained for the contaminated test of sea urchin 5 (red), for the contaminated gonads of sea urchin 5 (blue) and U-toposome complex (black)

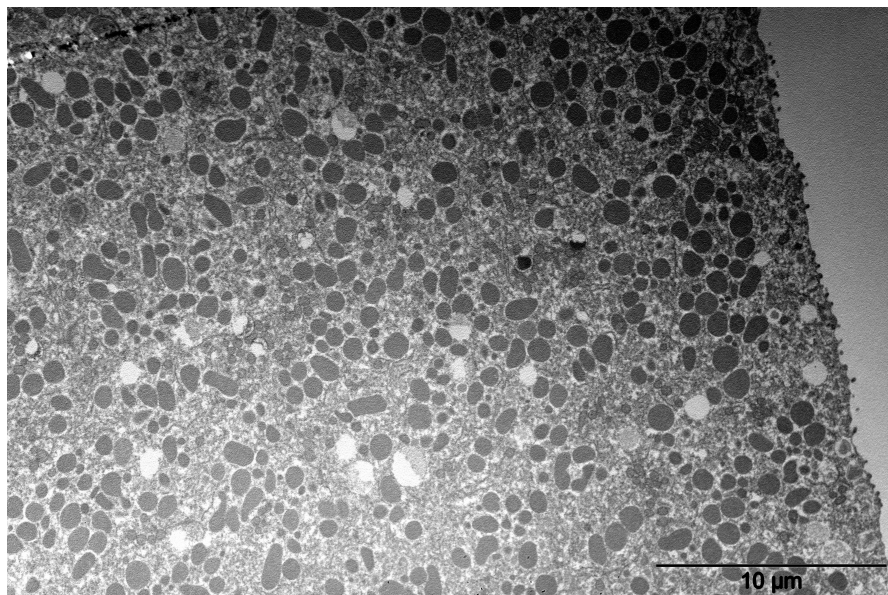


Figure 5: TEM imaging performed on the gonads of sea urchin 2.

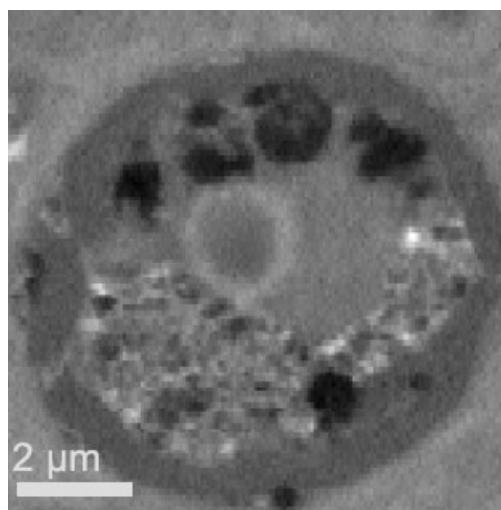


Figure 6: STXM normal contrast image of the sea urchin 2 gonad cells collected at 738 eV.

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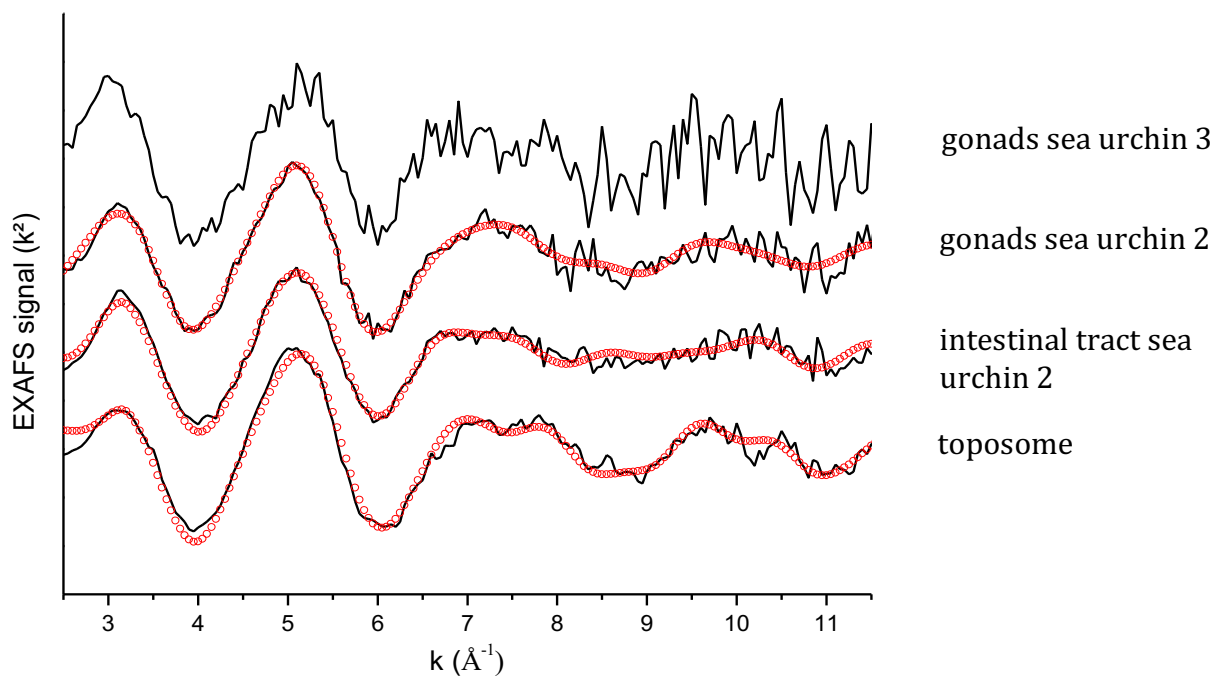
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Figure 7 : EXAFS spectra (k^2) at the U L_{III} edge of the contaminated intestinal tract of sea urchin 2 (SU2) and the U-toposome complex. The EXAFS spectra obtained for the contaminated gonads of sea urchin 3 (SU3) is also presented. Experimental = straight line; fit = red dots.

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