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Effects of uranium uptake on transcriptional responses, histological structures and survival rate of the crayfish *Procambarus clarkii*

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ABSTRACT

This work aims to investigate the accumulation levels and effects (transcriptional responses, histopathology and survival rate) associated with a wide range of dissolved uranium (U) concentrations (0, 0.03, 0.6, 4 and 8 mg/L of U) on adult male crayfish *Procambarus clarkii* during 4 (T4) and 10 (T10) days of exposure. The follow-up of the crayfish mortality showed that *P. clarkii* was highly resistant to U. Increasing waterborne U concentrations led to increasing bioaccumulation in key crayfish organs and increasing histological damages. U distribution in tissues was also evaluated using transmission electron microscopy and showed the presence of a detoxified form of U in the gill's epithelium in the shape of flakes. Expression levels of mitochondrial genes (*cox1*, *atp6* and *12S* gene) and genes involved in oxidative stress (*sod*(*Mn*) and *mt*) were examined together with the house-keeping gene 18S. *atp6* and *mt* genes of *P. clarkii* were cloned and sequenced before analysis. Significant correlations were observed between U bioaccumulation and the down-regulation of both *cox1* and *sod*(*Mn*) genes. This work provides a first U toxicogenomic and histopathological pattern of *P. clarkii*, identify U biomarkers and associate gene expression endpoints to accumulation levels. It also provides new insights into the mechanisms involved in U stress.

1. Introduction

Uranium's (U) distribution in ecosystems is currently increasing due to anthropogenic activities such as mining, milling, agricultural use (e.g. phosphate fertilizers), industrial activities linked to nuclear fuel production, and laboratory research activities (ASTDR, 1999; Colle et al., 2001; WHO, 2001). U concentrations in natural waters generally vary from below 12 ng/L up to 2 mg/L (Betcher et al., 1988) and may reach an excess of 10–20 mg/L in extremely polluted aquatic systems (Ragnarsdottir and Charlet, 2000). It is therefore essential to understand the potential impact of U on ecosystem components so that measures can be taken to protect the environment.

Procambarus clarkii is the most cosmopolitan crayfish around the world, is able to tolerate extreme and polluted environments, and has been used as an indicator of metal pollution in numerous studies of aquatic environments (Gherardi, 2006). Crayfish tend to accumulate metals (Alcorlo et al., 2006; Anderson et al., 1997; Sánchez López et al., 2004; Suárez-Serrano et al., 2010) such as U in their tissues (Chassard-Bouchaud, 1982; Simon and Garnier-Laplace, 2005). In addition, *P. clarkii* meets other criteria that made it suitable for toxicology and risk assessment studies for many decades. For example, it is relatively static, easily captured and adults provide sufficient tissue for individual analyses. Furthermore, its central position in aquatic food webs makes this species a potential vector of contaminants to higher trophic levels (Gherardi, 2006). It is also important to note that, in some countries, *P. clarkii* is a species of great commercial interest (Sánchez López et al., 2004).

U toxicity has not been extensively studied in non-human biota, especially in aquatic invertebrates such as crayfish. In order to address this deficiency, the proposed approach in this study is to evaluate the impacts of uranium on *P. clarkii*. The No Observed Effect Concentration (NOEC) data for aquatic species such as algae, micro-crustaceans, hydra and fish range from 18 to 810 μg U/L (Hogan et al., 2010). However information regarding the concentration of U required to cause an impact on crayfish is scarce. Thus, this ongoing research involves the investigation of the effects associated with a wide range of dissolved U concentrations extending from environmental relevant concentrations to dose rates found in extremely polluted sites (0.03–8 mg/L) on adult male crayfish *P. clarkii* during 10 days of exposure.

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Contaminants exert their toxicity at all levels of biological organization ranging from molecular levels to ecosystems (Perceval et al., 2006). Consequently, transcriptional responses using quantitative real-time RT-PCR, histopathological effects, survival rate, bioaccumulation levels and metal distribution using Transmission Electron Microscopy coupled with Energy Dispersive X-ray (TEM-EDX) were assessed as U endpoints. The investigation of U toxicity was conducted on gills and hepatopancreas of crayfish. Indeed, gills are the first biological barriers to suffer from heavy metal intoxication via a water exposure (Meyer et al., 1991; Niyogi and Wood, 2004) and hepatopancreas has a well-defined role in the storage, metabolism and detoxification of a number of metals (Alcorlo et al., 2006; Lyon and Simkiss, 1984; Muriana et al., 1993). Pollutants could impair mitochondrial function (Cannino et al., 2009; Garceau et al., 2010; Lerebours et al., 2010; Pourahmad et al., 2006; Risso-De Faverney et al., 2004), which could lead to disease, ranging from subtle alterations in cell function to cell death and from minor to major disability, or to death (Duchen, 2004a, b). Therefore, the expression level of the *cox1* gene encoding for the cytochrome c oxidase subunit 1 (complex IV) was examined in both organs. This enzyme is involved in the mitochondrial respiratory chain and helps to establish a transmembrane gradient of protons. The *atp6* gene encoding for ATP synthase subunit 6 (complex V) was cloned and sequenced before analyzing its expression in organs. This enzyme uses the gradient of protons created by the other complexes to synthesize ATP (Wang and Oster, 1998). The expression level of the mitochondrial gene *12S*, encoding for the ribosomal RNA 12S was used as an indicator of the number of mitochondria in cells (Renault et al., 2008). Moreover, examination of *sod*(*Mn*) gene expression level encoding for mitochondrial superoxide dismutase, an enzyme involved in the fight against oxidative stress (Wang et al., 2004) was conducted. The expression levels of *mt* gene were also determined after cloning and sequencing. This gene encodes for the protein metallothionein, which is known to be involved in detoxifying excess intracellular metals (Amiard et al., 2006; Baudrimont et al., 1999) and in the protection of cells against Reactive Oxygen Species (ROS) (Amiard et al., 2006; Viarengo et al., 2000). The objectives of this study were to comprehend the mechanisms of U toxicity, identify sensitive biomarkers of U exposure and clarify the relationships between U accumulation and endpoints at different levels of biological organization.

2. Materials and methods

2.1. General experimental protocol

The crayfish used in this study were all adult intermoult males (27.2 ± 5 g; 9 ± 0.5 cm from cephalothorax to telson; $n=100$). Organisms were caught on the same day from the Vigueirat swamp in Camargue, south of France (GPS coordinates: 43°31.863′N–4°45.417′E). They were acclimatized to the experimental conditions for one month (12/12 h light/dark photoperiod, and in 17 ± 1 °C synthetic water composed of: $Ca^{2+}=1640$; $Mg^{2+}=500$; $Na^{2+}=870$; $K^{+}=80$; $Cl^{-}=4100$; $SO_4^{2-}=509$; $NO_3^{-}=76.5$; and $HCO_3^{-}=281$ all in µmol/L; pH 6.7 ± 0.2). Freshwater ionic constitution was based on the composition of artificial medium used by Zeman et al. (2008) for the microcrustacean *Daphnia magna*. The synthetic water was modified to meet the physiological needs of crayfish. The chosen pH value and the voluntary lack of phosphate ensured high levels of U bioavailability. During the acclimatization period, crayfish were fed every 48 h on a diet of corn and mussels and then starved for 48 h before the beginning of the experiment, in order to avoid fecal matter and uneaten food to bind to U and therefore decrease metal bioavailability. During the experiment, animals were exposed for 10 days to a wide range of U concentrations: 0 (controls), 0.03, 0.6, 4 and 8 mg/L of U (from DU stock solution: $UO_2(NO_3)_2$, $6H_2O$, 1 g/L). The ionic concentrations of the medium (mainly NO_3^{-}) were adjusted for each condition of exposure to remain constant after U addition. U speciation in contaminated waters was simulated using the geochemical speciation software J-CHESS (Java Chemical Equilibrium with Species and Surfaces, Van der Lee, 1998). Groups of 10 crayfish were placed in each treatment tank with 6 L of test solution and so were submerged to 3 times their height following ASTM E729-96 recommendations. Half of the water column was renewed manually each day in order to maintain stable water composition and contamination levels. The concentrations of U in water were measured before and after renewal and adjusted to assure mean values significantly close to nominal contamination levels (Table 1). The ASTM standard guide lines for conducting acute toxicity tests on macroinvertebrates were respected (ASTM E729, 1996). Thus, the temperature was maintained at 17.2 ± 0.4 °C. Several abiotic parameters were monitored daily throughout the exposure, including dissolved oxygen ($73.6 \pm 12.4\%$ O_2), pH (7.01 ± 0.24), conductivity (602.4 ± 25.9 µS) and water concentration of ions (NO_3^{-}, NO_2^{-}, Cl^{-}, SO_4^{2-}, PO_4^{3-} by ICS-3000; Ion Chromatography System). The ionic concentrations did not differ significantly from the optimum concentrations mentioned above. Each crayfish was kept in an individual chamber (cylinder made from plastic netting (1 cm mesh, 11 cm diameter) to avoid cannibalism. Crayfish mortality was regularly checked each day.

2.2. Tissues sampling and preparation

At days 4 (T4) and 10 (T10), 4–5 live crayfish were sampled from each tank and sacrificed (Table 1). The hepatopancreas and gills were collected from each individual and divided into 3 parts. Two parts were stored at −80 °C for further bioaccumulation and genetic analyses. The third part was directly prepared for microscopic analyses.

2.3. Metals quantification

Water samples from each tank were acidified using nitric acid (3.1 mM of HNO_3) prior to analysis of metal content using inductively coupled plasma-atomic emission spectrometry (ICP-AES Optima 4300DV, PerkinElmer, Wellesley/USA; detection limit: 10 µg/L ± 10%).

Hepatopancreas and gill samples were first dried at 45 °C for 2 days, then digested in 3 ml of HNO_3 (15.5 M) for 90 min at 95 °C, and then left to dry for 60 min at 105 °C. 2 ml of H_2O_2 (33%) were added afterwards to complete the digestion and left to dry again for 20 min at 105 °C. Samples were finally dissolved in acidified water (3.1 mM of HNO_3) before analysis. Samples were analyzed for the highest U concentrations using ICP-AES and inductively coupled plasma mass spectrometry (ICP-MS VARIAN 810-MS; detection limit: 10 ng/L ± 7%) for the lowest concentrations. Some samples were analyzed using both ICP-AES and ICP-MS techniques to confirm that there were no significant differences between both analytical methods.

2.4. Histochemical and histological methods

Fresh tissues were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 48 h at 4 °C (Lerebours et al., 2010), then washed 3 times for 5 min with 0.1 M sodium cacodylate buffer (pH 7.4), and then post-fixed with 1% osmium tetroxide in the same buffer for 1 h. Samples were subsequently dehydrated with successive baths of ethanol and propylene oxide, and finally embedded in Epon 812 (TAAB). Samples were cut into semi-thin sections of 200–500 nm for light microscopy analysis. Semi-thin sections stained with aqueous blue toluidine were observed under a light microscope (Leica, DM 750) and images were saved using a Leica camera ICC50 and LAS EZ software. Ultra-thin sections of 80 and 140 nm were performed for Transmission Electron Microscope (TEM) observation and Energy Dispersive X-ray (EDX) detection, respectively. All sections were obtained using an ultramicrotome (UCT, Leica). Ultra-thin sections were mounted on copper grids and observed with a Scanning Transmission Electron Microscope (TEM/STEM, Tecnai G2 Biotwin, FEI company), equipped with a CCD camera (Megaview III, Eloise). Several different sub-cellular structures were analyzed using the EDX analyzer equipped with a Super Ultra Thin Window (SUTW) model sapphire (EDAX), using an accelerating voltage of 100 KeV, in order to investigate the accumulation of U in the different structures. The electron probe was then focused on specific spots and spectra were recorded after 30 s of analysis. For each replicate organ, at least 30 micrographs of local detailed structures were taken.

2.5. Genetic analyses

2.5.1. Total RNA extraction and reverse transcription of RNAs

Total RNA were extracted from 20 to 40 mg of tissue using the "Absolutely RNA Miniprep kit" (Agilent), according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (3 µg) using the Stratascript First-Strand Synthesis System (Agilent) according to the manufacturer's instructions. The cDNA mixture was stored at −20 °C until needed.

Table 1
Nominal and measured U (mg/L) concentrations in water throughout the experiment ($n=20$). U bioaccumulations (μg/g DW, mean ± SEM, $n=4$–5 in each treatment condition) in gills and in hepatopancreas of the crayfish *Procambarus clarkii* after 4 and 10 days exposure to U (0.03 to 8 mg/L).

Nominal U concentrations (mg/L)	Measured U concentrations (mg/L)	U bioaccumulation in gills (μg/g DW)		U bioaccumulation in hepatopancreas (μg/g DW)	
		T4	T10	T4	T10
0	0	0.30 ± 0.15 ($n=5$)	0.56 ± 0.10 ($n=5$)	0.29 ± 0.05 ($n=5$)	0.15 ± 0.01 ($n=5$)[d]
0.03	0.033 ± 0.003	152.87 ± 27.7 ($n=4$)	129.96 ± 9.00 ($n=4$)	2.84 ± 0.60 ($n=4$)[a]	1.15 ± 0.60 ($n=4$)[d,*]
0.6	0.630 ± 0.060	603.90 ± 94.00 ($n=4$)	570.94 ± 107.40 ($n=5$)	6.73 ± 2.00 ($n=4$)[ab]	10.48 ± 2.80 ($n=5$)[e]
4	3.95 ± 0.14	3950.50 ± 770.00 ($n=5$)[a]	8483.49 ± 2250.00 ($n=5$)[b]	21.43 ± 9.30 ($n=5$)[bc]	14.13 ± 4.00 ($n=5$)[ef]
8	8.34 ± 2.5	8005.30 ± 2370.00 ($n=4$)[a]	12,345.74 ± 5403.00 ($n=5$)[b]	30.39 ± 14.00 ($n=4$)[c]	78.78 ± 31.20 ($n=5$)[f,*]

[a,b,c,d,e,f] Means of each sampling time designated with the same letters are not significantly different ($P>0.05$).
* Significant difference between the bioaccumulation at day 4 (T4) and day 10 (T10) ($P<0.05$).

Table 2
Gene names, primers deduced from alignment of corresponding sequences from different species and their accession numbers.

Gene	Primers (5′–3′)	Accession number
ATP synthase subunit 6 (*atp6*)	CGATTGGCAGCAAATATAATTGC[a] ATGATTGCAACGGCAGATTCTA[b]	*Cherax destructor* AY383557 *Pseudocarcinus gigas* NC006891 *Cherax setosus* AY153876 *Panaeus notialis* X84350 *Euphausia superba* AB084378
Metallothionein (*mt*)	GAGTGTGCCGAGGGCGGGTG[a] GCAGGGCTTGGAGCAGGTCTTG[b]	*Portunus pelagicus* AY057395 *Homarus americanus* AJ401298 *Pacifastacus leniusculus* AF199482

[a] Forward primer.
[b] Reverse primer.

Table 3
Gene names, accession numbers and specific primers pairs used in the quantitative PCR analysis of the 6 studied genes of *P. clarkii*.

Gene	Primers (5′–3′)	Accession number
Cytochrome C oxidase subunit 1 (*cox1*)	AATGGGATACCTCGACGTTATTCA[a] GCAGGAGGATAAGAATGCTGT[b]	AY701195.1
ATP synthase subunit 6 (*atp6*)	GGCAGCAAATATAATTGCTGG[a] TTGCAACGGCAGATTCTAATAT[b]	GU220369.1
Ribosomal RNA 12 S (*12S*)	ACTAGAATATTAGGAGTTATGTTCTT[a] GCTGCACCTTGATCTAATATAC[b]	EF012280.1
Manganese Superoxide dismutase *sod(Mn)*	GCCACCACTAAAATACGAGTA[a] CCATTGAACTTTATAGCTGGTA[b]	EU254488.3
Metallothionein (*mt*)	CGAGGGCGGGTGCAAGACT[a] CTTGGAGCAGGTCTTGGCAC[b]	GU220368.1
18S ribosomal RNA (*18s*)	GCAATAACAGGTCTGTGATGCC[a] AGGGACGTAATCAGCGCAA[b]	X90672.1

[a] Forward primer.
[b] Reverse primer.

2.5.2. Cloning and sequencing of mt and atp6 genes

To obtain a partial coding sequence for metallothionein and ATP synthase subunit 6, primers were deduced from alignment of corresponding sequences available in libraries from different phylogenetically related species (preferentially crustaceans) using the Clustalw software (Table 2). These primers deduced from conserved sequences, were then used on cDNA in PCR experiments (50 amplification cycles at 95 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min). PCR products were cloned into pGEM-T-easy Vector System (Promega) with T4 DNA ligase. Competent Cells (Promega) were transformed with 3 μl of the ligation mixture. Positive transformants were selected on ampicillin-containing medium (Luria Broth: 5 g/L of yeast extract, 10 g/L of bactotryptone, 10 g/L of NaCl supplemented with 100 μg/mL ampicillin) and recombinant vectors were then purified and sequenced. Partial genes sequences were determined by Millegen Biotechnologies (La-bège, France) and were validated by comparing their similarity to other genes in GeneBank using Blastp and Blastn software (National Center of Biotechnology Information, Bethesda, MD, USA). *mt* was registered in GenBank under the accession number GU220368.1 and *atp6* under GU220369.1.

2.5.3. Real-time quantitative PCR

Real-time PCR reactions were performed in a Light Cycler (Roche) following the manufacturer's instructions using the DNA intercalating dye SybrGreen I. The amplification program consisted of one cycle at 95 °C for 10 min followed by 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s. Specific primer pairs used to determine target gene expression levels are listed in Table 3. PCR specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SybrGreen fluorescence level during a gradual heating from 60 to 95 °C. Relative

quantification of each gene expression level was normalized according to the expression of the housekeeping gene *18S* gene. Relative expression of a gene was calculated using the $2^{-\Delta CT}$ method as described by Livak and Schmittgen (2001) where ΔCT represents the difference between the cycle threshold of a specific gene and the cycle threshold of the *18S* gene. Therefore, the Expression Factor (EF) of each gene compared with control corresponds to the following equation: $EF = 2^{-\Delta CT}(\text{Treatment})/2^{-\Delta CT}(\text{Control})$.

2.6. Statistical analysis

Results are given as means ± the Standard Error of the Mean (SEM). All analyses were done using the R language and environment for statistical computing (R Development Core Team, 2009). For all statistical results, a probability of $p < 0.05$ was considered significant. Survival rate was calculated using Kaplan–Meier's estimator (Kaplan, 1983). This method estimates the fraction of living organisms for a certain period of time after treatment and takes into account "censored" data (i.e. crayfish lost due to sampling at T4 before the final outcome was observed at T10). To determine whether there is or not a significant effect of contaminant concentrations on survival rates, a regression using Weibull distribution was performed. The significant effects of factors such as time and metal concentrations in water on bioaccumulation were analyzed using Analysis of Covariance (ANCOVA), after checking assumptions of normality and variance homogeneity of residuals. The assumption of normality was checked visually using a Quantile–Quantile plot (QQplot) of standardized residuals *versus* normal quantiles. The assumption of variance homogeneity was checked on residuals using standardized residuals *versus* a fitted plot (Faraway, 2002). When the assumptions were not satisfied, a logarithmic data transformation was applied. If significant effects were detected, Tuckey's multiple comparison test was used to determine whether means between pairs of samples were significantly different from one another. The relationships between metal concentration in organs, and gene expression levels of each individual were investigated using the non-parametric Spearman rank correlation test due to the non-linearity of the data.

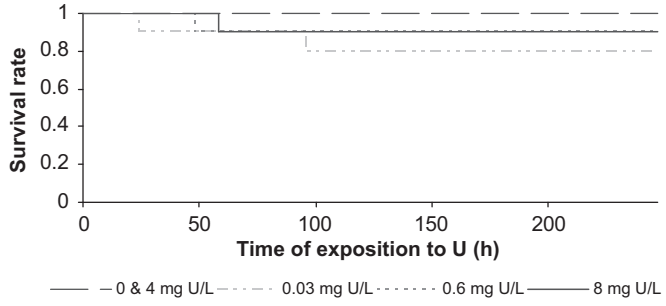

Fig. 1. Kaplan–Meier's presentation of the survival rate of *Procambarus clarkii* exposed to U during 240 h of exposure.

3. Results

3.1. Survival rate

No mortality was recorded in the control group (0 mg/L). Increasing U concentrations had no significant effect on crayfish survival ($P = 0.0794$; Fig. 1). 100% survived exposure to 4 mg/L of U at T10 whereas 80% survived exposure to 0.03 mg/L of U. After 10 days of exposure to 0.6 and 8 mg/L of U, only 10% of the crayfish died.

3.2. U bioaccumulation in crayfish organs

The results of U accumulation in the gills and hepatopancreas at T4 and T10 are shown in Table 1 and expressed in dry weight (DW). Within the control group, U levels were less than 0.6 μg/g DW. As U concentrations in water rose, accumulation of the metal in the gills rose from 100 to 12,000 μg/g, and accumulation of U in the hepatopancreas ranged from 1 to 80 μg/g. A high correlation was found between U bioaccumulation in organs and the concentrations of U in the test water (gills: Spearman coefficient $R = 0.94$, $P < 2.2 \times 10^{-16}$; hepatopancreas: Spearman coefficient $R = 0.88$, $P = 3.47 \times 10^{-15}$). At each time tested U concentrations in gills were significantly different when compared between each other ($P < 0.05$), except between the concentrations in gills exposed to 4 and 8 mg/L of U ($P = 0.2$ at T4 and $P = 0.92$ at T10). A dose dependency of U bioaccumulation was also observed in the hepatopancreas. U accumulation was not significantly time dependent ($P = 0.069$) in gills, yet there was a significant difference between U concentrations in the hepatopancreas of crayfish exposed to 0.03 and 8 mg/L of U ($P < 0.05$) at T4 and T10. Concentrations of U were considerably higher in gills than in hepatopancreas at T4 as well as at T10 (T4: $P = 4.9 \times 10^{-13}$; T10: $P = 1.5 \times 10^{-6}$).

3.3. U localization and histological alterations

Histological localization of U using TEM-EDX showed flake shaped residues of U, co-localized with phosphorus in the gill epithelium after 10 days of exposure (Fig. 2). Distinct alterations of the tissue structure could be discerned best in the hepatopancreas after 10 days of contamination (Fig. 3). The epithelium of several tubules showed numerous pathologically altered cells

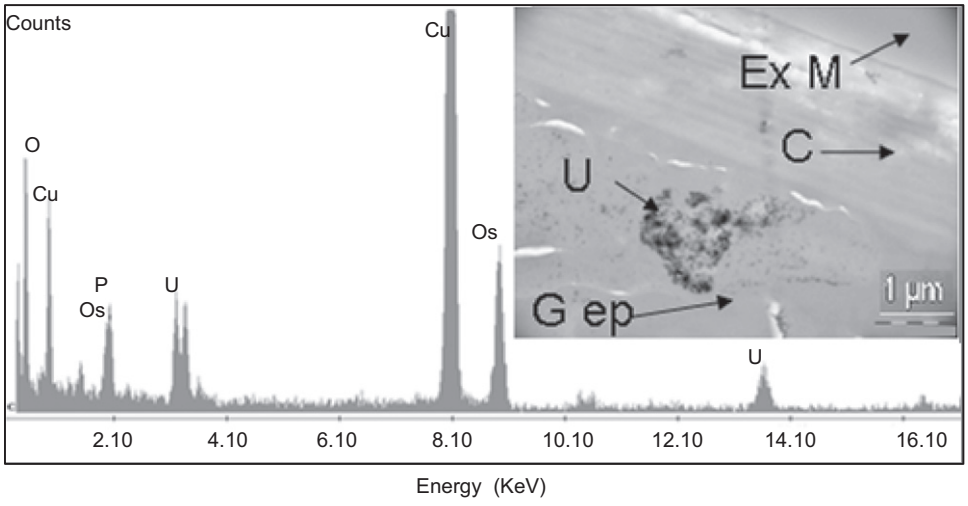

Fig. 2. Transmission electron micrographs coupled with energy dispersive X-ray results of *P. clarkii* gills exposed for 10 days to 4 mg/L of U. Elements detected in matrix: O: oxygen, Os: osmium and Cu: copper. Elements detected in gills epithelium: U: uranium and P: phosphorus. (Ex M) extra-cellular matrix, (C) cuticle and (G ep) gills epithelium.

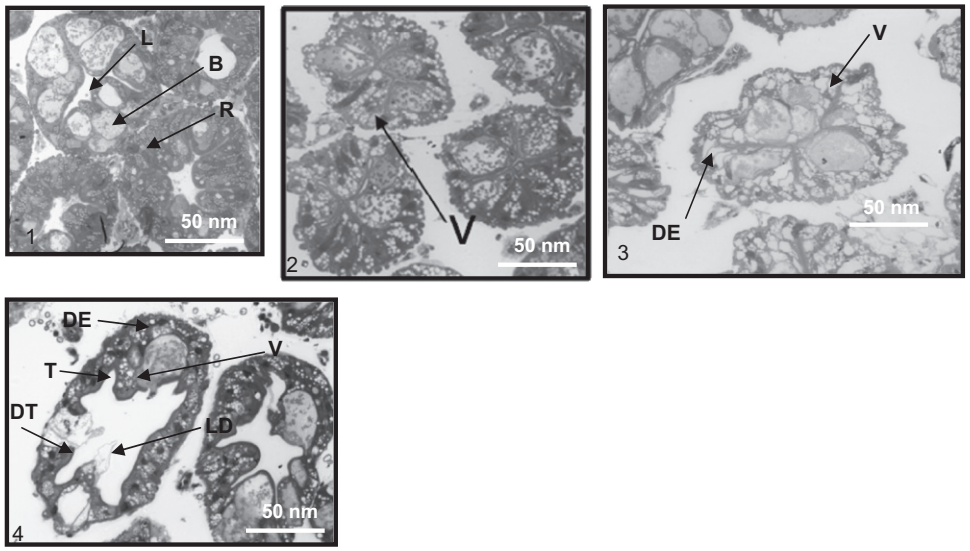

Fig. 3. Histopathological alterations in *P. clarkii* hepatopancreas tubules after 10 days of exposure to dissolved heavy metals: (1) control; (2) 0.6 mg/L U; (3) 4 mg/L U; (4) 8 mg/L U. (R) absorptive cells. (B) Secretory cells. (L) Lumen. The pathologies observed were: (V) vacuolization; (T) thinning of the epithelium; (LD) lumen dilatation; (DE) disorganized epithelium; (DT) degenerated tubule. Magnification × 40.

Table 4
Expression's Factors (EF) of the 5 genes studied in *P. clarkii* compared to the basal level of controls (n=4–5 in each treatment condition).

Organs	U treatment condition (mg/L)	*cox1*		*atp6*		*12S*		*sod*(*Mn*)		*mt*	
		T4	T10	T4	T10	T4	T10	T4	T10	T4	T10
Gills	0.03	/	/	55	/	−2	/	−3	/	−3	3
	0.6	/	−3	3	/	−7	/	−2	2,5	−5	17
	4	/	−2	/	/	−7	/	/	6	−4	32
	8	−3	−2	/	3	−10	−2,5	/	/	−6	5
Hepatopancreas	0.03	/	−7,5	64	/	2	/	/	−5	2	/
	0.6	/	/	/	10	/	/	/	/	/	/
	4	2	/	/	/	2	−2,5	/	−3	/	/
	8	−5	/	/	/	/	−3,5	−2	/	/	/

(/): Equal to control. (−): Down-regulated. (+): Up-regulated. Only EF ≥ 2 or EF ≤ −2 are considered statistically significant.

with cytoplasmic vacuoles. Epithelial organization of cells was lost because cell walls were disintegrated. Degenerated tubules were also observed at high levels of U contamination. However, this phenomenon was confined to individual tubules and did not affect the entire digestive gland, so some intact glandular epithelium was present even at high concentrations of exposure.

3.4. Gene expression levels

In contaminated gills, the *cox1* gene was repressed tardily in the three highest U conditions (Table 4). The *atp6* gene was over-expressed at T4 at the two lowest U exposure levels, and at T10 when exposed to 8 mg/L of U. The expression levels of the *12S*, *mt* and *sod*(*Mn*) genes decreased at T4 and then increased at T10.

Contaminated hepatopancreas showed the most pronounced changes at the lowest U concentration tested. Indeed, at 0.03 mg/L the expression levels of *atp6*, *12S* and *mt* were increased at T4 but returned to basal level at T10. In contrast, *cox1* and *sod*(*Mn*) remained unchanged at T4 and were repressed at T10. No significant variations were observed for most of the genes in the hepatopancreas when exposed to 0.6 mg/L, except for the *atp6* gene at T10. At 4 mg/L an over-expression was observed at T4 for *cox1* and *12S*, while reduced expression levels appeared at T10 for the *12S* and the *sod*(*Mn*) genes. At the highest U exposure level, only *cox1* and *sod*(*Mn*) were repressed at T4, as well as *12S* at T10.

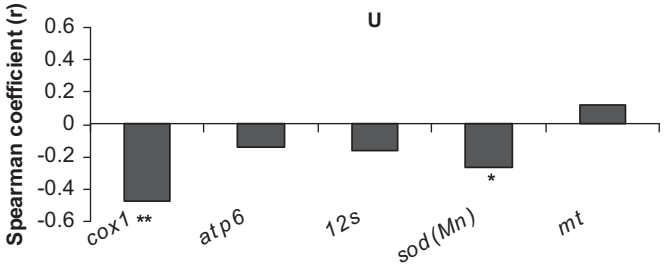

Fig. 4. Spearman correlation coefficients (r) between U concentrations in organs and gene expression levels of the 5 genes studied (n=4–5 in each treatment condition). (*) $P<0.05$. (**) $P<0.005$.

3.5. Gene expressions vs. bioaccumulation

A correlation between gene expression levels and metal bioaccumulation was assessed without taking into account any possible variations in molecular responses, which might depend on the type of organs and the period of exposure (Fig. 4). Thus, for every sampling time, the bioaccumulation of U in each organ of each individual was associated with the genes expression levels, which were pertaining in that same organ. In both studied organs the *cox1* gene expression level was negatively correlated with U concentration in organs (Spearman coefficient $R=-0.48$, $P=1.2\times10^{-5}$). Moreover, the down-regulation of *sod*(*Mn*) appeared

to be highly correlated with U bioaccumulation in gills and hepatopancreas (Spearman coefficient $R=-0.3$, $P=0.021$).

4. Discussion

4.1. Effects on survival rate

The follow-up of the crayfish mortality showed that *P. clarkii* was highly resistant to U toxicity since no significant mortality was observed even at the highest concentrations of exposure. The 10 days U LC_{50} in our experimental conditions (pH 7) is probably higher than 8 mg U/L and confirms the resistance capacity of this species to the tested metal. Few ecotoxicological data concerning the effects of U on aquatic invertebrates are available in the literature, and to our knowledge the U LC_{50} values of crustaceans are limited to those of micro-organisms (Poston et al., 1984; Kuhne et al., 2002; Sheppard et al., 2005; Zeman et al., 2008). Some laboratory studies have shown that the toxicity of U decreases as pH, conductivity and alkalinity of the water increases (Markich et al., 2000; Poston et al., 1984; Zeman et al., 2008). Zeman et al. (2008) confirmed that the 48h-LC_{50} of *D. magna* varied from 0.390 ± 0.040 mg/L at pH 7 to 7.8 ± 3.2 mg/L at pH 8. The U 48-hr LC_{50} of this same species in Columbia River water (with high levels of organic matter) was 6 mg/L at pH 7.9–8. Acute toxicity diminished by a factor of 7.5 as mean water hardness and alkalinity values increased (Poston et al., 1984). The bioavailability and toxicity of uranium are closely linked to its chemical speciation in solution. Metal uptake and toxicity normally vary as a function of the concentration of the free-metal ion in solution (Niyogi and Wood, 2004). The influence of pH is twofold: on one hand, increasing pH results in enhanced complexation of the uranyl ion by hydroxides and carbonates, hence reducing its bioavailability; on the other hand, the decrease in competing protons can increase uranyl bioavailability (Fortin et al., 2007). These considerations led us to conduct our experiments in synthetic water, which provides a compromise between the conditions necessary for healthy crayfish physiology and good U bioavailability. The capacity of crayfish to tolerate exposure to high levels of U could allow the use of this species as a bioindicator of a large range of contamination levels.

4.2. Accumulation

Elevated concentrations of U in organs were observed despite the low mortality rate. It is difficult to understand how high levels of U can be tolerated by crayfish without increased mortality without knowing how toxic metals are distributed at the subcellular level in their tissues (Perceval et al., 2006). Our TEM observations led to the detection of piles of flake shaped U inside the gill epithelium. This form of U was mainly co-localized with phosphorus, which is insoluble and non-toxic. U detoxification mechanisms have been described by Chassard-Bouchaud (1982) in the crayfish *Pontastacus leptodactylus* and in the bivalve *Corbicula fluminea* by Simon et al., 2011. These authors found that U was stored in spherocrystals and in lysosomes as a complex with phosphates. Granules containing heavy metals have been found in epithelial cells of invertebrates. These granules may contain either calcium or heavy metals such as zinc, copper or iron, complexed with sulfur or phosphorus (Ahearn et al., 2004). Identical results have been observed in *Astacus astacus* after direct exposure to aluminum (Fjeld et al., 1988). Subsequent cellular exocytotic events may extrude these concretions from the cell as part of an excretory mechanism (Ahearn et al., 2004; Marigómez et al., 2002). However, in the case of radionuclides, high levels of U (an alpha emitter) even in precipitated forms could have radiotoxic effects. U bioaccumulation in organs was dose-dependent since U concentrations in tissues were highly correlated with those in water (Spearman coefficient $R=0.9$ in gills; $R=0.8$ in hepatopancreas; $P<0.05$). There was a tendency for metal bioaccumulation to increase with time both organs. Crayfish were able to rapidly bioconcentrate the contaminant in their tissues (high BCF: e.g. BCF gills-8 mg U/L$\sim$832). Nevertheless, metal concentrations in the gills were higher than those in the hepatopancreas. Moreover, the bioaccumulation tended to stabilize after exposure to 4 mg/L of U. The gills are in direct contact with the medium, which normally results in both adsorption and internalization of toxic metals. Adsorption of U on the cuticle could explain the high accumulation levels, even though the MET and EDX analyses did not show such a mechanism. High metal concentrations in water increase the occurrence of interactions between U and conveyors located in the gills epithelium, so it is possible that a high metal adsorption occurred and could have saturated the sites of entrance of the metal resulting in a stabilization of the bioaccumulation.

4.3. Histopathological effects

The histological observations revealed information about U effects on another level of biological organization and helped better understand the toxicological profile of U. The extent of the histopathological damage was in line with the bioaccumulation of U determined in the soft tissues and appeared to be dose-dependent, as evidenced by the increasing vacuolization and the tendency of the epithelium to disintegrate, and the tubules to degenerate. U exposure induces a variety of histological impairments in fish (Barillet et al., 2010; Cooley and Klaverkamp, 2000). The whitefish *Coregonus clupeaformis* showed significantly altered hepatocyte morphometrics, hepatocyte necrosis and alterations of bile ductile epithelium when fed U-contaminated diet (Cooley and Klaverkamp, 2000). Vacuolization in hepatic cells of the juvenile northern pike *Esox lucius* resulting from contamination with U have also been reported (Kelly and Janz, 2009). Even though U concentrations in our experiment did not lead to significant mortality of crayfish, severe histological damages were observed and could be used as biomarkers of U contamination.

4.4. Molecular responses

The study of gene expression levels helps understanding uranium's mechanisms of toxicity and then enables the determination of good biomarkers of contamination. The molecular responses suggested that mitochondria were affected by the presence of U in both organs. Gene expression levels were less altered in hepatopancreas than in gills, this could be due to the lower levels of U in the hepatopancreas. U had a considerable effect on the cytochrome c oxidase (complex IV) since a significant relationship was observed between U concentrations and *cox1* gene repression (Fig. 4). The correlation between U concentrations in organs and *sod*(*Mn*) gene repression was also dose-dependent. Moreover, the down-regulation of *cox1* gene was significantly correlated with *sod*(*Mn*) (Spearman coefficient $R=0.6$; $P<0.001$). It is well known that in the absence of any contamination, mitochondria are the main source of ROS in the cell (Wang et al., 2004). Thus we can hypothesize that with the impairment of the mitochondrial metabolism, ROS levels decreased, which led to the repression of *sod*(*Mn*) gene. Recent experiments on zebrafish *Danio rerio* have shown that the presence of extremely low concentrations of U in the organs could also result in an impact on the expression of genes involved in resisting oxidative stress and in mitochondrial metabolism (Lerebours et al., 2010; Lerebours et al., 2009). Over-expression of the *atp6* gene could be explained by two different mechanisms. The first possible explanation is that

mitochondria produced more ATP synthase to create enough ATP to maintain cell viability and compensate for the impairment of the complex IV. However in the literature it is more common to find that the gene expression of *atp* and *cox* follows the same pattern. For example, in the eel *Anguilla anguilla* Cd caused a decrease in both *cox1* and *atp6–8* genes expression levels when exposed for 14 days to 2 and 10 μg/L of Cd (Pierron et al., 2007). At high levels of exposure to U, a second explanation for the increase of the expression levels of *atp6* gene could be linked to a change in the classic mitochondrial metabolism. It is known that in some pathological conditions, the complex V can show a "reverse" mechanism. When the complex IV is inhibited, the proton transfer from the mitochondrial matrix to the periplasmic space decreases. A severe decrease of the proton gradient leads to a decrease of the mitochondrial inner membrane potential which stimulates the functioning of ATPase in an unusual manner. In this case, the complex V can reverse itself and hydrolyze ATP to pump protons back to the periplasmic space (Nevière, 2008; Wang and Oster, 1998). We can therefore hypothesize that the mitochondria produced enough ATPase to compensate for the decrease of the electrochemical gradient. Expression levels of the *12S* gene indicated that cells were struggling against the toxicity of U by increasing the numbers of mitochondria to make up for those which were not functioning correctly. Metallothioneins are characterized by an important proportion of cysteinyl residues, which serve as ligands for metal chelation (Amiard et al., 2006; Martín-Díaz et al., 2006) but can also trap Reactive Oxygen Species (ROS) (Fang et al., 2010; Viarengo et al., 2000). The *mt* gene was more expressed at T10 than T4 in all exposure conditions. This could be linked to the natural presence of a sufficient concentration of MT proteins at the beginning of the exposure. In the presence of large quantities of MT (ex T10), cells could increase the numbers of their mitochondria since this protein can efficiently trap the ROS. Consequently cells could reduce their effort in producing ATP synthase (*atp6* less up-regulated at T10). The significant up-regulation of the *mt* gene in the gills at T10 demonstrates that U can generate an important oxidative stress when the organs present high U accumulations. These results agree with previous studies that have shown oxidative stress in fish after exposure to waterborne U (Barillet et al., 2007; Barillet et al., 2011). Information on MT response in the presence of U is scarce. Cooley and Klaverkamp (2000) found no increase of MT concentration in the liver of U-contaminated fish except for the highest trophic exposure (10 mg U/g) at day 10. A recent study showed that in the presence of MTs the percentage of nematode *Caenorhabditis elegans* mortality, decreased when exposed to different concentrations of depleted U (Jiang et al., 2009). However, the exact mechanism by which MTs afford nematodes protection to U exposure remains unclear.

5. Conclusions

Mitochondria appear to be cellular targets for U. The impact on these organelles was evident from the alteration of mitochondrial genes expression levels. Impairment of the mitochondrial metabolism was accompanied by alterations in the expression levels of genes involved in responses to oxidative stress responses and was followed by histological damages. The study of gene expression levels could be a promising tool for evaluating stress due to U exposure. *P. clarkii* bioaccumulated U rapidly and was highly resistant to the contamination. It could therefore be a good candidate for use as a bioindicator of U pollution and in ecological risk assessments. Evaluation of U concentrations in sub-cellular fractions is also necessary to accurately highlight the distribution of U inside the cell. Further research is also needed to better understand the impairment of the mitochondrial chain reaction. Measurements of ATP levels, oxygen consumption, ROS production in the cells and evaluation of enzymes activities involved in oxidative stress responses could clarify the effect of U on ATPsyntase.

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