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Effects of uranium on crayfish *Procambarus clarkii* mitochondria and antioxidants responses after chronic exposure: What have we learned?

Simone Al Kaddissi [a,b], Alexia Legeay [b], Antonia Concetta Elia [c], Patrice Gonzalez [b], Virginie Camilleri [a], Rodolphe Gilbin [a], Olivier Simon [a,*]

[a] Laboratory of Radioecology and Ecotoxicology (LRE), Institute of Radioprotection and Nuclear Safety (IRSN), Bd 186, BP 3, 13115 Saint-Paul-Lez-Durance, France
[b] Laboratory of Aquatic Ecotoxicology, University Bordeaux1/UMR CNRS 5805, Dr Peyneau square, 33120 Arcachon, France
[c] Ecotoxicology Laboratory, Department of Cellular and Environmental Biology, University of Perugia, 06123 Perugia, Italy

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ABSTRACT

We examined the impacts of Uranium (U) on mitochondria and on the response of antioxidants in the gills and the hepatopancreas of crayfish *Procambarus clarkii* after long-term exposure (30 and 60 days) to an environmentally relevant concentration (30 μg U/L). The expression of mitochondrial genes (*12s*, *atp6*, and *cox1*), as well as the genes involved in oxidative stress responses (*sod(Mn)* and *mt*) were evaluated. The activities of antioxidant enzymes (SOD, CAT, GPX and GST) were also studied. U accumulation in organs induced changes in genes' expression. The evolution of these transcriptional responses and differences between gene expression levels at high and low doses of exposure were also discussed. This study demonstrated that, after long-term exposure, U caused a decrease in antioxidant activities and induced oxidative stress. A possible ROS-mediated U cytotoxic mechanism is proposed. Expression levels of the investigated genes can possibly be used as a tool to evaluate U toxicity and seem to be more sensitive than the enzymatic activities. However a multiple biomarker approach is recommended as the perturbed pathways and the mode of action of this pollutant are not completely understood.

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1. Introduction

Uranium (U) is a ubiquitous environmental trace metal, often found in water supplies as a non-essential inorganic component (Bleise et al., 2003). Concentrations in freshwater ecosystems are highly variable and range from 0.01 μg/L to over 12.4 mg/L, depending on the geological background (Salonen, 1994; WHO, 2001). U may occur in different oxidation states, the major forms being U(VI) in oxic water, and U(IV) in anoxic water (Markich, 2002). In addition to its natural occurrence and distribution in the environment, U has also several civilian and military applications that could cause its dispersion and increase its abundance in environmental compartments (Bleise et al., 2003). These anthropogenic activities include the use of phosphate fertilizers, various mining activities, and the industrial processing of U — including the use of depleted U — for the manufacture of nuclear fuel and other products (ATSDR, 1999). Depleted U (DU) is artificially obtained as a by-product of the U enrichment process and is about 60% less radioactive than natural U, which is considered a weakly radioactive element. Depleted U, however, retains all the chemical properties of natural U (WHO, 2001). Thus, effects from exposure of biota to either natural U or DU are usually attributed to their chemical toxicity (ATSDR, 1999). Nevertheless, the mechanisms by which this metal induces its toxicity have not been sufficiently investigated (Pourahmad et al., 2006; Barillet et al., 2007; Al Kaddissi et al., 2011). A greater knowledge of interactions between U and living organisms is needed in order to select pertinent biomarkers that could be used in ecological risk assessments. Certain studies have shown that this radioelement is able to chemically activate oxygen species in the course of redox reactions via the redox chemistry of transition metals (Miller et al., 2002; Yazzie et al., 2003). Moreover, Jones et al. (2003) stated that since U is an alpha-emitting radioactive element (WHO, 2001; Taulan et al., 2004) it can enhance the production of free radicals via the ionization phenomenon induced by alpha particle emissions. High quantities of free oxygen species (generated within cells) sometimes exceed the cell's protective controllability, resulting in damage to cell proteins, nucleic acids and lipids (Labrot et al., 1996; Barillet et al., 2007; Lourenço et al., 2010). In a previous study, carried out in our laboratory, we demonstrated that U alters the expression of some mitochondrial genes (*atp6*, *cox1*, *12S*) and genes involved in oxidative stress responses (*sod(Mn)*, *mt*) in *Procambarus clarkii* after short-term exposure (4 and 10 days) to 30 μg/L (Al Kaddissi et al., 2011). We supposed that this radioelement can generate oxidative stress. Further experiments were,

* Corresponding author. Fax: +33 4 42199151.
E-mail address: olivier.simon@irsn.fr (O. Simon).

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however, required to improve our understanding of the effects of U on *P. clarkii* and to choose potential markers of U toxicity. Thus evaluation of the responses of the selected biomarkers after long term exposure was needed. It was also necessary to investigate the responses of the classic major indicators of oxidative stress such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST). Since the genes of *P. clarkii* that encode for these antioxidants have not been sequenced, the investigation of oxidative stress induction had to be done by following the enzymatic activities. It is established that *P. clarkii* is a cosmopolitan species (Gherardi, 2006), relatively static, easily captured, bioaccumulates U (Al Kaddissi et al., 2011) and adults provide sufficient tissue for individual analyses. The use of this species as a biological model is therefore of great interest. In our current work the expression levels of mitochondrial genes (*12s, atp6* and *cox1*) and genes involved in oxidative stress responses (*sod(Mn)* and *mt*) were evaluated and the enzymatic activities of antioxidants (SOD, CAT, GPX and GST) were studied after 30 and 60 day exposure to 30 μg U/L. The concentration of U that we selected is within the range commonly found close to drill wells (Kurttio et al., 2006) and mining sites (e.g. 20 μg/L) (Simon and Garnier-Laplace, 2005). It is also double the World Health Organization provisional drinking water guideline (15 μg/L) (WHO, 2004) but equal to the recommended levels of the USEPA promulgated in 2000 (EPA 2000). Gills and hepatopancreas were collected after various periods of exposure to assess the different biological parameters and U bioaccumulation levels were studied in parallel. Comparisons between genes expression levels after short-term (4 and 10 days) (Al Kaddissi et al., 2011) and long-term exposures, and after high and low levels of contamination, were conducted. This approach helps identify possible changes in the mechanisms of action of U over time, and helps gather information to choose appropriate biomarkers of U contamination. The opportunity was also taken to link the antioxidant responses to the mitochondrial dysfunction.

2. Materials and methods

2.1. General experimental protocol

Adults inter-molt *P. clarkii* males were used in this study (23.55 ± 1 g fresh weight; 8.96 ± 0.11 cm from cephalothorax to telson; $n=30$). These were obtained from the Vigueirat swamp of Camargue, France (GPS coordinates: 43°31.863′N–4°45.417′E).

Crayfish were acclimatized to experimental conditions for 3 weeks under a 12/12 h light/dark photoperiod at 17 ± 1 °C while submerged in synthetic water equilibrated by means of air bubbling (water composition: $Ca^{2+}=1640$; $Mg^{2+}=500$; $Na^{2+}=870$; $K^{+}=80$; $Cl^{-}=4100$; $SO_4^{2-}=509$; $NO_3^{-}=76.5$; $HCO_3^{-}=281$ all in μmol/L; pH 6.6 ± 0.3). The artificial water composition, the pH value (6.5) and the voluntary lack of phosphate were chosen as a compromise to ensure abiotic conditions that satisfied the physiological needs of crayfish (Al Kaddissi et al., 2011) and to optimize U bioavailability (Fortin et al., 2007; Fortin et al., 2004). Speciation of 30 μg/L of U in the synthetic water at pH 6.5 was simulated using the geochemical speciation software J-CHESS (Java Chemical Equilibrium with Species and Surfaces, Van der Lee, 1998). Based on this simulation 0.7% of UO_2^{2+} and 4.6% of UO_2OH^{+} were present in the medium (Table S1). These forms of U are known to be the most bioavailable species to aquatic organisms (Markich, 2002, Fortin et al., 2004, 2007). The dominant species under these conditions were $(UO_2)_2CO_3(OH)_3^{-}$ (42%) and UO_2CO_3(aq.) (25.7%), which can be accumulated by organisms (Denison, 2004). Commercial trout pellets were fed to crayfish, at dosages of 0.1 g/individual, every 2 days. Nourishment continued during exposure conditions. During the acclimation and experimentation phases, animals were kept at a maximum density of 3 crayfish/L. Groups of 10 crayfish were exposed to 0 (control) and 30 μg U/L for 60 days. The stock solution of U used to contaminate water was prepared from depleted uranyl nitrate $UO_2(NO_3)_26H_2O$ (Sigma, France) and acidified with nitric acid (0.016 M). 25% of the water column was renewed daily by a continuous flow-through technique. A large volume of each test solution (0 and 30 μg/L) was prepared once a week and maintained at a constant flow through the respective tanks containing crayfish. U concentration in the water column was measured once daily and adjusted, if necessary, to ensure constant contamination pressure. Temperature and pH were monitored daily, whereas NO_3^{-}, NO_2^{-}, Cl^{-}, SO_4^{2-} and PO_4^{3-} concentrations were verified twice a week by ICS-3000, Ion Chromatography System. Crayfish were isolated in individual chambers (plastic netting: 1 cm mesh, 11 cm diameter) to avoid cannibalism. Five crayfish were sampled from each tank at Day 30 (T30) and Day 60 (T60). The hepatopancreas and gills of each individual were collected, split into three parts and stored at −80 °C for further bioaccumulation, gene expression and enzymatic activities' analyses.

2.2. Uranium quantification

Water samples were acidified by nitric acid (3.1 mM of HNO_3) prior to metal quantification by inductively coupled plasma-atomic emission spectrometry (ICP-AES Optima 4300DV, PerkinElmer, Wellesley/USA; detection limit: 10 μg/L $\pm$ 10%). Organs were dried at 45 °C for 2 days and, then digestion was performed in a DigiBLOC 3000 digestion system (SCP Science, Champlain, NY, USA). Thereafter, 3 mL of 15.5 M nitric acid was added to each organ and was heated for 90 min at 95 °C after which samples were evaporated in a second heating cycle (60 min, 105 °C). The digestion process was then completed by the addition of 2 mL H_2O_2 (33%) followed by evaporation (20 min, 105 °C). Samples were dissolved in acidified water (3.1 mM of HNO_3) prior to analysis. ICP-AES was used to determine the highest U concentrations and inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500 Cs; detection limit: 10 ng/L $\pm$ 7%) to determine the lowest concentrations. Combined tests, using ICP-AES and ICP-MS techniques, were also run to validate the assumption that there was no significant difference between results from these two analytical methods.

Table 1
Genes, accession numbers, specific primers pairs used in the quantitative PCR analysis of the 6 studied genes of *P. clarkii* and their function.

Gene	Primers (5′–3′)	Function and accession number
cox1*:* Cytochrome C oxydase subunit 1 (complex IV).	AATGGGATACCTCGACGTTATTCA[a] GCAGGAGGATAAGAATGCTGT[b]	Encoding for an enzyme in the inner membrane of mitochondrion that helps establish a transmembrane gradient of protons. AY701195.1
atp6: ATP syntase subunit 6 (complex V).	GGCAGCAAATATAATTGCTGG[a] TTGCAACGGCAGATTCTAATAT[b]	Encoding for an enzyme in the inner membrane of mitochondrion that uses the gradient of protons created by other complexes to synthesize ATP. GU220369.1
12S: Ribosomal RNA 12 S.	ACTAGAATATTAGGAGTTATGTTCTT[a] GCTGCACCTTGATCTAATATAC[b]	Indicator of the amount of mitochondria in cells. EF012280.1
Sod(Mn): Mitochondrial Mn- Superoxyde dismutase.	GCCACCACTAAAATACGAGTA[a] CCATTGAACTTTATAGCTGGTA[b]	Encoding for an enzyme involved in the fight against oxidative stress. EU254488.3
mt: Metallothionein.	CGAGGGCGGGTGCAAGACT[a] CTTGGAGCAGGTCTTGGCAC[b]	Encoding for MT protein involved in detoxifying Cd and in the protection against reactive oxygen species. GU220368.1
18s: 18S ribosomal RNA.	GCAATAACAGGTCTGTGATGCC[a] AGGGACGTAATCAGCGCAA[b]	Housekeeping gene. X90672.1

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

2.3. Total RNA extraction, reverse transcription of RNAs and real-time quantitative PCR

Total RNA was extracted from gills (20–40 g samples) or hepatopancreas ($n=5$ for each experimental condition and sampling time) using the "Absolutely RNA Miniprep" kit (Agilent). First-strand cDNA was synthesized from total RNA using the Stratascript First-Strand Synthesis System (Agilent). First-strand cDNA was synthesized from total RNA using the AffinityScriptTM Multiple Temperature cDNA Synthesis Kit (Stratagene). In each of the above three tests, the techniques were carried out according to the manufacturer's instructions. The cDNA mixture was stored at -20 °C, until required. The accession numbers of the studied genes and their function are listed in Table 1. For each gene, specific primer pairs were determined using the LightCycler probe design software (Ver 1.0; Roche). The amplification of cDNA was monitored, using the DNA intercalating dye SyberGreen I (Roche). Real-time PCR reactions were performed in a Light Cycler (Roche). The amplification program consisted of one cycle at 95 °C for 10 min and 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s. This dissociation curve was obtained by following the SyberGreen I fluorescence level during a gradual heating from 60 to 95 °C. For each amplification, a single peak was observed for all the primer-pairs used, indicating the amplification of a single product. The amplified cDNAs in the first set of experiment were analyzed by gel electrophoresis to verify that amplified fragments were of the expected molecular weight. Results validated the PCR primers' specificity. Primer efficiencies were determined for each quantitative PCR run by a dilution series. Efficiency was always between 90% and 102%, as classically expected. Relative quantification of each gene expression level was normalized according to the 18S gene expression, a housekeeping gene. 18S gene was chosen as the reference gene because of its stability over time and treatment in the experiment. Relative expression of a gene was generated 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. The comparison of the expression's factor (EF) of each gene with the control therefore corresponded to the following equation: $EF=2^{-\Delta CT}(\text{Treatment})/2^{-\Delta CT}(\text{Control})$.

2.4. Enzyme activity

Enzyme activity was determined in cellular cytosolic fractions of tissues to assess possible links between cellular oxidative stress and the dysfunction of mitochondria in the presence of U. The gills and hepatopancreas of each individual were thus, respectively, homogenized, in 1:10 and 1:20 weight:volume (W/V) proportions, in ice cold Tris–HCl buffer (100 mM, pH 7.8) containing 100 μM phenylmethanesulfonyl fluoride (PMSF), 0.008 aprotinin U/ml and 0.1 mg/ml of bacitracin. An aliquot of each homogenate was centrifuged at 15,000*g* for 30 min at 4 °C; supernatants were further centrifuged at 100,000*g* for 1 h at 4 °C. All biochemical analyses were performed with a SOFTmax PRO spectrophotometer at a constant temperature of 25 °C. Total protein contents of gills and hepatopancreas (S100 fractions) were determined using the "Micro BCA^{TM} Protein Assay Kit" (Thermo scientific) according to the manufacturer's instructions. Absorbance was read at 562 nm using a plate reader. CAT activity was measured by following the decrease in absorbance at 240 nm due to H_2O_2 consumption ($\varepsilon=0.04$ mM^{-1} cm^{-1}). The assay took place in a Na-phosphate buffer (100 mM, pH 7) and 12 mM H_2O_2. Superoxide dismutase SOD was determined spectrophotometrically using the "19160 SOD determination kit" (Fulka) according to the manufacturer's instructions. Absorbance was read at 450 nm using a plate reader. SOD activity was expressed as U/mg protein (Prot). GPX activity towards cumene hydroperoxide as a substrate was determined and oxidation of NaDPH was followed at 340 nm ($\varepsilon=6.22$ mM^{-1} cm^{-1}). The assay condition was as follows: 100 mM Na-phosphate buffer with pH 7.5, 1 mM EDTA, 0.12 mM NaDPH, 2 mM GSH, 1 mM DTT, 0.8 mM cumene hydroperoxide and 1 U of glutathione reductase. GST activity was measured using 1-chloro-2.4-dinitrobenzene (CDNB) as a substrate and the formation of the conjugate with GSH was followed at 340 nm ($\varepsilon=9.6$ mM^{-1} cm^{-1}). The assay condition was as follows: 100 mM Na-phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CDNB.

2.5. Statistical analysis

Results are given as means ± the standard error of the mean (SEM). Statistical analysis was performed using the R language and environment for statistical computing (R Development Core Team, 2009). The significant effects of certain factors (such as time and metal concentration in water) on bioaccumulation or on enzyme activity were estimated using analysis of variance (ANOVA) when normality and variance homogeneity assumptions were satisfied. The normality assumption was checked using a quantile–quantile plot (QQplot) of standardized residuals versus normal quantiles. The variance homogeneity assumption was checked on residuals using standardized residuals versus fitted plot (Faraway, 2002). When the assumptions were not satisfied, a logarithmic data transformation was applied. If the assumptions were still not satisfied, Kruskal–Wallis non-parametric ANOVA was used. To assess the link between U concentrations in water and in organs, the correlation coefficient (R) was calculated using Spearman rank correlation test. The same test was also applied to compare gene expression levels after short and long periods of exposure. Transcriptional responses in contaminated organs in the previous study (Al Kaddissi et al., 2011) and in the current study were correlated with varying exposure periods (4, 10, 30 and 60 days). A probability of $P<0.05$ was considered significant for all statistical results.

3. Results

3.1. Experimental conditions

Mortality was not observed during acclimation and metal exposure periods. Physicochemical parameters such as temperature (16.84 °C ± 0.06), pH (pH = 6.24 ± 0.04) and concentrations of major anions (which were significantly identical to desired concentrations) remained stable. The measured U concentration in water was equal to 28 ± 0.8 μg U/L ($n=60$) and thus not significantly different ($P>0.05$) from the nominal concentration (30 μg/L).

3.2. Uranium bioaccumulation

The Uranium concentrations in water were strongly correlated to tissue concentrations in organs (gills: $R=0.87$, $P=7\times10^{-6}$; hepatopancreas: $R=0.87$, $P=3.9\times10^{-7}$). Concentrations of U in organs of the control crayfish were extremely low and did not exceed 0.13 μg/g of dry weight (DW) (Table 2). A significant difference was discerned between U concentrations in contaminated organs and control animals ($P_{gills}=1.43\times10^{-5}$; $P_{hepatopancreas}=4.71\times10^{-6}$). Bioaccumulation in these organs in exposed crayfish did not, however, differ significantly over time. U accumulation levels were eight and four times higher in gills than in the hepatopancreas at T30 and T60, respectively.

3.3. Gene expression after long term exposure

Transcriptional responses after long term exposure are presented in Table 3. In gills, the genetic analysis indicated a decrease in the number of mitochondria at T30 and T60 as *12S* gene expression levels decreased. A change in the functioning of the mitochondrial chain reaction was observed by following gene

Table 2
U bioaccumulation (μg/g dry weight (DW), mean ± SEM, $n=5$ in each treatment condition) in gills and in hepatopancreas of the crayfish *Procambarus clarkii* exposed at day 30 (T30) and day 60 (T60) to 0 (control) and 30 μg/L of U.

Nominal U concentrations in water (μg/L)	U bioaccumulation in gills (μg/g DW)		U bioaccumulation in hepatopancreas (μg/g DW)	
	T30	T60	T30	T60
0	0.13 ± 0.01	0.11 ± 0.02	0.05 ± 0.01	0.06 ± 0.01
30	165.70 ± 22.15	155.35 ± 30.89	20.11 ± 6.99	39.91 ± 8.07

Table 3
Expression's factors (EF) of the 5 studied genes in gills and hepatopancreas of *P. clarkii* compared to the basal level of controls ($n=5$ in each treatment condition) at day 30 (T30) and day 60 (T60).

Organs	*12s*		*atp6*		*cox1*		*sod(Mn)*		*mt*	
	T30	T60	T30	T60	T30	T60	T30	T60	T30	T60
Gills	−4.5	−2	−88	8	/	2	−3	4	−2	−3.4
Hepatopancreas	−3.5	2.5	/	3	4	3	3	3	/	2

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

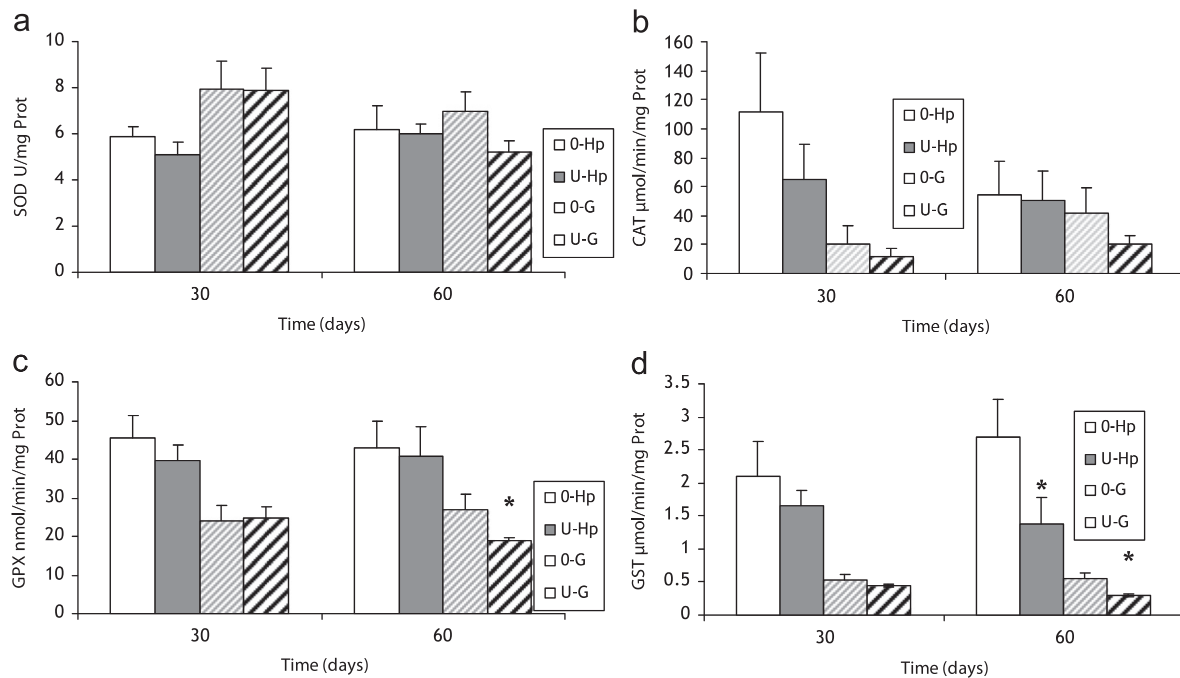

Fig. 1. Enzymatic activities of superoxide dismutase (SOD) (U/mg Prot, mean ± SEM, $n=5$ in each treatment condition), catalase (CAT) (µmol/min/mg Prot, mean ± SEM, $n=5$ in each treatment condition), glutathione peroxidase (GPX) (nmol/min/mg Prot) and glutathione S transferase (GST) (µmol/min/mg Prot, mean ± SEM, $n=5$ in each treatment condition) in gills and in hepatopancreas of the crayfish *Procambarus clarkii* exposed for 30 and 60 days to 0 (control) and 30 µg/L of U. The symbol (*) indicates significant statistical differences between the control and treated samples ($P<0.05$). (0-Hp)—control hepatopancreas. (U-Hp)—contaminated hepatopancreas. (0-G)—control gills. (U-G)—contaminated gills.

expression levels of both *atp6* and *cox1* genes. The expression of the mitochondrial gene *atp6* was altered in the presence of U as this gene was highly down-regulated at T30 (−88-fold) and then up-regulated at T60 (8-fold). Gene expression of *cox1* was only up-regulated at the end of the experiment in gills of contaminated crayfish. Expression levels of the *sod(Mn)* gene seemed to increase with time: this gene was repressed 3-fold at T30 but was up-regulated 4-fold at T60. The *mt* gene was repressed in the gills of crayfish exposed to U during both experimental periods.

Expression levels of the *12S* gene in the hepatopancreas decreased about 3.5-fold at T30, then increased 2.5-fold up at T60. Moreover, the *atp6* gene was only 3-fold up-regulated at T60, while the *cox1* and *sod(Mn)* genes were up-regulated in this organ at both times. No differences were noted between the expression of the *mt* gene in contaminated hepatopancreas and controls at T30. Nevertheless, this gene was 2-fold up-regulated at T60.

3.4. Enzyme activity

No significant statistical variation in the activity of either SOD or CAT was discerned in contaminated organs when compared to controls (Fig. 1). A tendency of these enzymes to deplete can, however, be observed in exposed gills at T60. Crayfish exposed to U exhibited a significant depletion (30 ± 3.4%) in GPX activity in gills at T60, when compared to the control. A significant decrease in GST activity was also discerned in organs at the end of the experiment at levels of about 46 ± 3.76% and 48.6 ± 14.33% in the gills and hepatopancreas, respectively.

4. Discussion

4.1. Bioaccumulation

Significant concentrations of U in the gills (accumulation factor up to 6) and the hepatopancreas of crayfish were observed at T30 and T60, despite the low level of U contamination in water. Al Kaddissi et al. (2011) recorded that bioaccumulation levels of U in gills of *P. clarkii* were equal to 152.87 ± 27.7 µg U/g DW at T4 and to 129.96 ± 9 µg U/g DW at T10 after exposition to 30 µg U/L *via* a direct route. Equivalent results were obtained for concentration levels in the same organ at T30 and T60. U bioaccumulation in gills thus seemed to rapidly reach an equilibrium state. As expected, gills accumulated more U than hepatopancreas at T30 and T60. Such results were also observed in an earlier study when Al Kaddissi et al. (2011) noted that the bioaccumulation of U in gills was around 50 to 100 times greater (at T4 and T10, respectively) than that recorded in the hepatopancreas of *P. clarkii*. Contamination of the crayfish *Orconectes limosus* to U *via* a trophic route led to significant accumulation of the metal in the hepatopancreas (up to 20 µg/g fw), which was up to 20 times higher than that recorded in gills (Simon and Garnier-Laplace, 2005). Thus the type of exposure (direct/trophic) influences the distribution of U in organs. U concentrations in the hepatopancreas at T30 (20.11 ± 6.99 µg U/g DW) and T60 (39.91 ± 8.07 µg U/g DW) were elevated but no statistical significant difference between values was noted. These U concentrations were much higher than those reported in the hepatopancreas at T4 (2.84 ± 0.6 µg U/g DW) and T10 (1.15 ± 0.6 µg U/g DW) (Al Kaddissi et al., 2011). It thus seems that there is a significant effect of time on the bioaccumulation of U in the heparopancreas, when comparing the concentration levels in the organ after short- and long-term exposure.

4.2. Transcriptional responses after long-term exposure to U

The repression of *12S* gene in gills at T30 and T60 indicates a decrease in the number of these organelles in U-contaminated cells. Such effects, relating to U toxicity, were also observed in gills of *P. clarkii* exposed to high U concentrations (0.6, 4 and 8 mg/L) after 4 days of contamination, when the *12S* gene was noted to have been down-regulated by 7- to 10-fold (Al Kaddissi et al., 2011). Some heavy metals are known to induce structural

damage to mitochondria, resulting in a destruction of cristae and the outer or inner mitochondrial membranes (Triebskorn and Köhler, 1996; Lei et al., 2011). Structural damage induced by U could have thus led to a decline in the number of mitochondria in contaminated organs. Gough (1931) described that mitochondria in renal epithelium of U-contaminated rabbits had formed small granules or were replaced by large spherical bodies that were scattered in the cytoplasm. The *cox1* gene was only 2-fold up-regulated at T60 in gills. Likewise, this gene expression levels in gills of *Danio rerio*, exposed to 23 ± 6 μg/L of U for 28 days, did not vary significantly when compared to control fish (Lerebours et al., 2009). Conversely, the *cox1* gene was reported to be down-regulated (2 to 3-fold) in gills of *P. clarkii* presenting high levels of U bioaccumulation (Al Kaddissi et al., 2011). The expression of this gene thus appears to be modulated by the level of U concentration in tissues. Lerebours et al. (2010) stated that the intensity of gene response may not correlate positively with toxicant concentrations and that different gene expression patterns are expected for different concentrations of exposure. This statement is in concordance with our findings in terms of comparing the gene response of *cox1* after acute exposure to that of chronic exposure to U, which indicates that a modification in the metabolism might occur in organs presenting different levels of contamination. The over-expression of *cox1* in the present study also coincided with an increase in expression of the *atp6* gene in gills at the end of the experiment. The up-regulation of these two genes indicates an increase in mitochondrial metabolism. This could be a cellular strategy to compensate for the decrease in the number of functional mitochondria by increasing ATP production, so as to provide enough energy for cellular needs. It was also noted that gene expression levels of *sod(Mn)* correlated with those of *cox1* at T30 and T60 (in gills: $R=0.4$; in hepatopancreas: $R=0.5$; $P=0.05$). In our previous work, we observed a significant correlation between these two genes' expressions ($R=0.6$, $P<0.001$) in organs of crayfish exposed to 0.03, 0.6, 4 and 8 mg/L of U for 10 days (Al Kaddissi et al., 2011). The results of these two studies confirmed that when mitochondrial metabolism increases in the presence of U, the endogenous ROS production increases and, consequently, the mitochondrial antioxidant defense mechanism is induced. On the contrary, when the mitochondrial metabolism decreases, the over-expression of genes encoding for antioxidants does not seem necessary. This cellular response to U toxicity was also observed in the hepatopancreas at T30 and T60. The *cox1* and *sod(Mn)* genes were also reported to be up-regulated, by 5- and 11-fold, respectively, in the liver of *Danio rerio* exposed to 23 ± 6 μg/L of U during 28 days (Lerebours et al., 2009). Another interesting response, involving *12S* gene expression, was observed in the contaminated hepatopancreas. In effect, the amount of mitochondria decreased significantly at T30 and then increased at T60, accompanied by an increase in mitochondrial metabolism (*atp6* and *cox1* up-regulated) in this organ at the end of the experiment. This indicates that cells are able to adopt various compensatory mechanisms in response to toxicity, including an increase in the amount of mitochondria. The expression of the *mt* gene was repressed at T30 and T60 in gills whereas it was 2-fold over-expressed in the hepatopancreas at T60. The response of metallothionein protein (MT) to U contamination is not well understood. An *in vitro* study by Michon et al. (2010) demonstrated that U can bind to MT, while a study by Cooley and Klaverkamp (2000) demonstrated that, in living organisms such as fish, no increase in MT was observed in contaminated livers, except in animals that had been subjected to the highest trophic exposure (10 mg U/g- at day 10). An increase in *mt* gene expression in highly contaminated gills of *P. clarkii* was reported after 10 days of direct exposure to U (0.6–8 mg/L). No such increase was noted in the hepatopancreas of the same animal, which presented low levels of U bioaccumulation (Al Kaddissi et al., 2011). Moreover, MT is known to bind ROS efficiently (Viarengo et al., 2000; Fang et al., 2010), which leads to the assumption that this protein participates in the antioxidant defense system particularly in highly contaminated tissues. In the current study, we hypothesized that the basal pool of MT in gills was sufficient and did not necessitate an over-expression of the corresponding gene with the actual concentration levels. In the hepatopancreas, the over-expression of the *mt* gene could be linked to oxidative stress. The increase of the amount of mitochondria (*12S* up-regulated), the augmentation of mitochondrial metabolism (*atp6* and *cox1* up-regulated), the induction of mitochondrial antioxidant defenses (*sod(Mn)* upregulated) and the depletion of cytosolic antioxidants, all indicated an increase in ROS levels in the organs at T60.

4.3. Evolution of transcriptional responses over time and their use as biomarkers

Expression of mitochondrial genes in gills and the hepatopancreas revealed a dysfunction in the energetic process after short-term (4 and 10 days) (Al Kaddissi et al., 2011) and long-term exposure periods (at T30 and T60) to 30 μg/L of U. Overall the *12S* gene was down-regulated over time in both organs (gills: $R=-0.45$, $P=0.052$; hepatopancreas: $R=-0.51$, $P=0.03$). This gene seems to be a sensitive biomarker of U contamination in gills since it was down-regulated soon after exposure (2-fold down-regulated at T4) (Al Kaddissi et al., 2011) and was also repressed after a long period of exposure (at T30 and T60). It should be noted, however, that (at T4 and T60) the *12S* gene was occasionally over-expressed in the hepatopancreas. Nonetheless, the expression levels of *atp6* gene fluctuated over time in both organs and did not follow a specific pattern, even though levels of U concentrations in the gills were identical at the different sampling times (at T4, T10, T30 and T60). Thus, this gene response seems to be modulated by the energetic demands of the tissue and not directly by U concentrations. The *cox1* and *sod(Mn)* gene expression levels tended to increase with time in both tissues (e.g.: hepatopancreas: $R=0.42$, $R=0.6$, respectively, $P<0.05$). These genes could thus be used as biomarkers of a long-term exposure to environmentally-relevant concentrations of U, which would be useful in monitoring programs. The *mt* gene, on the other hand, was generally repressed in gills ($R=-0.67$, $P=0.009$) but very little is known of the impact of U on MT. Consequently, the use of this response alone as a biomarker of U contamination is not sufficiently substantiated. Contrary to results obtained in gills, *mt* gene expression tended to increase in the hepatopancreas with time ($R=0.45$, $P=0.067$). It therefore appears that *mt* gene expression varies in different organs, which possibly relates to the kinetics of U exchange in different cellular compartments.

4.4. Oxidative stress responses after long term exposure to U

In the cytosolic fraction of contaminated organs, ROS levels were more likely to increase with time, given that the activity of antioxidants had a tendency to decrease. There is indeed evidence to indicate that oxidative stress, induced by metals in a biological system, can be attributed to alterations in the antioxidant defense system (Jemai et al., 2007). For example, it is known that cadmium indirectly induces oxidative stress by decreasing levels of antioxidants, which lead to an increase in levels of free radicals (Ercal et al., 2001; Watjen and Beyersmann, 2004; Newairy et al., 2007; Cao et al., 2010; Jomova and Valko, 2011). GPX activity decreased significantly in contaminated gills of *P. clarkii* compared to that measured at T60 in the control. An *in vitro* study (Labrot et al., 1996) indicated that such enzyme activity decreased significantly in fish

(*Brachydanio rerio)* that were in contact with 500 mg/L of U. These authors hypothesized that inactivation of enzymes could be a consequence of metals binding to the protein chain backbone. Recent research in U toxicology revealed that this metal can bind directly to some proteins, such as metallothioneins, ferritin, transferrin and albumin (Michon et al., 2010). To the best of our knowledge, no studies have been undertaken to examine the possibility of U binding to enzymes involved in the antioxidant defense system. A study by Blum and Fridovich (1985) demonstrated that superoxide radicals $O_2^{\cdot -}$ and hydroperoxides can deactivate GPX by converting its reduced form to the oxidized form, and that the presence of SOD prevents this inactivation. SOD catalyzes the conversion of reactive superoxide anions ($O_2^{\cdot -}$) to produce hydrogen peroxide (H_2O_2) (Ruas et al., 2008; Aschner and Jiang, 2009), which is subsequently dealt with by CAT (Kono and Fridovich, 1982; Michiels et al., 1994) and GPX activities (Michiels et al., 1994; Arthur, 2000). A decrease in SOD and CAT activities in contaminated tissues can thus increase the levels of $O_2^{\cdot -}$ and hydroperoxides, which in turn could cause an oxidative alteration of GPX, resulting in its loss of function. In the current study, the activities of SOD and CAT tended to decline with time, particularly in contaminated gills, which might contribute to a decrease in GPX activity. In addition, various studies showed that U causes a depletion of glutathione content in contaminated tissues (Pourahmad et al., 2006; Barillet et al., 2007; Periyakaruppan et al., 2007; Aschner and Jiang, 2009; Yapar et al., 2010; Pourahmad et al., 2011; Viehweger et al., in press). Thus non-protein thiol plays an important role in ROS scavenging, since it is a substrate for the removal of H_2O_2 by GPX. Consequently, a decrease in GSH content in tissues could lead to a decrease of GPX activity. GSH can also be conjugated to other proteins that are catalyzed by GST. GST transfers GSH groups to proteins to target for cellular export and subsequent metabolism and detoxification (Dringen, 2000; Dickinson and Forman, 2002; Hayes et al., 2005). Accordingly a decrease in GSH levels could explain a decrease in GST activity, observed in both of the contaminated organs of crayfish at T60. It is also possible that a decrease in antioxidant activity stems from the down regulation of the corresponding genes (Lerebours et al., 2009; Barillet et al., 2011).

4.5. Linking cellular oxidative stress to the dysfunction of mitochondria

A recent *in vitro* experiment in contaminated plant cells demonstrated a reduction, of about 33%, of U(VI) to U(IV) in the presence of GSH (Viehweger et al., in press). Pourahmad et al. (2006) also stated that the reductive activation of U(VI) by GSH to U(V) and finally U(IV) provides enough redox cycling to generate considerable amounts of ROS ($O_2^{\cdot -}$). Such results could explain the depletion of antioxidant activities observed in this study, since oxidation of enzymes renders them inactive (Davies, 2005). Pourahmad et al. (2006) also reported that U(VI) (50 μM) oxidized 85% of the GSH in rat hepatocytes, which resulted in the formation of oxidized glutathione (GSSG) and thus decreased the GSH content in cells. These findings reinforce the hypothesis that a depletion of GSH, as a substrate of GPX and GST, may have occurred in the current study, thus causing a decrease in the activity of these compounds. The reduction of antioxidant activities in the present study demonstrated that U generates oxidative stress, which is undoubtedly accompanied by an increase in ROS levels. It is well established that mitochondria are the primary source of ROS and the target of excessive ROS generation (Lei et al., 2011; Simon et al., 2000). It is also known that an exogenous source of ROS can cause oxidation of mitochondrial compounds, accompanied by direct local membrane perturbation (Zamzami et al., 1995). Pourahmad et al. (2006) found evidence for a collapse in mitochondrial membrane potential (MMP) of rat hepatocytes in the presence of U, which results in severe ATP depletion and mitochondrial permeability transition pore opening (MPT). The authors believe that mitochondrial damage is caused by ROS formation and that the collapse of MMP and MPT pore opening is the result of oxidation of thiol groups in the MPT pore region of the mitochondrial outer membrane and consequent conformational alteration. This suggestion is consistent with our results and could explain alterations in transcriptional responses in contaminated organs, particularly in gills at T60. An increase in *cox1* gene expression levels could be a response to remediate a possible decrease of the mitochondrial membrane potential. Moreover, the up-regulation of the *atp6* gene could be a strategy to compensate for a decrease in ATP levels, which is a consequence of mitochondrial damage caused by ROS, whereas the down-regulation of the *12S* gene indicates a loss of mitochondria, which could be the result of oxidative damage.

5. Conclusions and perspectives

In summary, U induces changes in mitochondrial genes expression levels and in the expression of genes involved in the antioxidant defense mechanism. Nonetheless, some transcriptional responses may differ between acute (Al Kaddissi et al., 2011) and chronic contamination, indicating that the mechanisms of U toxicity are different at low and high exposure concentrations. Moreover, when crayfish *P. clarkii* are exposed to environmentally-relevant concentrations of U, the expression of the studied genes may differ over time, indicating that responses are also time dependent. The *12S*, *cox1* and *sod(Mn)* genes seem to be sensitive biomarkers of U contamination. Nevertheless, a multi-biomarker approach is recommended for the evaluation of U toxicity because the mechanisms of action of this metal are not completely understood. This study also demonstrated that U induces oxidative stress and a loss of antioxidant response. A possible ROS-mediated U cytotoxic mechanism was also proposed. Moreover, it seems that the study of transcriptional responses is a more sensitive tool than the follow up of antioxidant activities for assessing U toxicity in crayfish. Further research is needed to better comprehend cytotoxic mechanisms associated with the presence of U. The measurement of glutathione (total, reduced and oxidized fractions) and the reduced form of U(IV) in tissues, as well as their correlation to enzymatic effects and ROS levels, could be of interest. The sequencing of cytosolic *sod(Cu)*, *cat*, *gpx* and *gst* is also needed in order to study their expression in contaminated tissues and to link possible alterations to enzymatic activities. This approach will partly help to elucidate the origin of the inhibition of the studied enzymes. We recommend *in vitro* assays, to ascertain whether U can bind directly to SOD, CAT, GPX and GST.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2011.11.026.

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