How Toxic is the Depleted Uranium to Crayfish *Procambarus clarkii* Compared with Cadmium?

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ABSTRACT: Due to a lack of information on the assessment of uranium's (U) toxicity, our work aimed to compare the effects of U on the crayfish *Procambarus clarkii* with those of the well documented metal: cadmium (Cd). Accumulation and impacts at different levels of biological organization were assessed after acute (40 μM Cd or U; 4–10 days) and chronic (0.1 μM Cd or U; 30–60 days) exposures. The survival rates demonstrated the high tolerance of this species toward both metals and showed that Cd had a greater effect on the sustainability of crayfish. The concentration levels of Cd and U accumulated in gills and hepatopancreas were compared between both conditions. Distinctions in the adsorption capacities and the mobility of the contaminants were suspected. Differences in the detoxification mechanisms of both metals using transmission electron microscopy equiped with an energy dispersive X-ray were also pointed out. In contrast, comparison between the histological structures of contaminated hepatopancreas showed similar symptoms. Principal component analyses revealed different impacts of each metal on the oxidative balance and mitochondria using enzymatic activities and gene expression levels as endpoints. The observation that U seemed to generate more oxidative stress than Cd in our conditions of exposure is discussed. © 2014 Wiley Periodicals, Inc. Environ Toxicol 31: 211–223, 2016.

Keywords: cadmium; uranium; survival rates; histology; antioxidants; mitochondria; crayfish; gene expression

INTRODUCTION

Trace metals are ubiquitous. If their environmental concentration exceeds a certain threshold value, they will adversely affect living organisms. Considering their biological effects, this group of contaminants is extremely heterogeneous. In fact, some trace metals such as cadmium (Cd) are toxic even in small quantities. Others such as uranium (U) have the distinctive feature of being radioactive (Ribera et al., 1996; WHO, 2001). Even though natural U is radiotoxic, it is believed that the major hazard rendered by this element results from its heavy metal toxicity rather than its radiological toxicity (Fisenne and Welford, 1986; Barillet et al., 2007; Al Kaddissi et al., 2012c). Depleted U (DU) is a low-level radioactive product which activity is about 60% lower than that of natural U (WHO, 2001). Hence, effects from exposure of biota to depleted or natural U are usually attributed to their chemical properties (ASTDR,). Due to a

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lack of information on the assessment of uranium's toxicity, we choose to compare the effects of Cd and U on the crayfish *Procambarus clarkii* after acute and chronic exposures using studies previously carried out in our laboratory (Al Kaddissi et al., 2011, 2012a, b). The goal of this work is to produce more reliable information on the toxicity of U by choosing a comparative ecotoxicological approach. This approach allows the collection of data on different effects and detoxification strategies developed by this species and point out differences or similarities in molecular or cellular damage caused by Cd or U. In addition, this comparative study also permits to distinguish which of these two metals is the most toxic to crayfish. It is known that U shares structural similarities with a number of different metals, primarily Cd and lead (Domingo, 1994; Bleise et al., 2003; Arfsten et al., 2005).

Groups of adult male crayfish were exposed to 0 and 40 μM of Cd or DU for 4–10 days (acute exposure) (Al Kaddissi et al., 2011, 2012b) while others were exposed to 0 and 0.1 μM for 30–60 days (chronic exposure) (Al Kaddissi et al., 2012a, b). The impact of the metals on the survival rates of crayfish were reported in each study. In addition, gills and hepatopancreas were collected after varying exposure periods (T4, T10, T30, and T60) to assess the impacts of Cd and U on different biological endpoints and metal bioaccumulation. The distribution of the metals in the epithelium of organs collected from acute exposure experiments was evaluated as well transmission electron microscopy-equiped with an energy dispersive X-ray (TEM-EDX). Possible effects on the histological structures of the hepatopancreas were also examined at T10 and T60 after acute and chronic contamination, respectively. The expression levels of mitochondrial genes (*12s, atp6*, and *cox1*) and genes involved in oxidative stress responses (*sod(Mn)* and *mt*) were also studied in all experiments and finally the enzymatic activities of antioxidants (SOD, CAT, GPX, and GST) were reported after chronic exposure to U and Cd (T30 and T60). New results are also presented in this study to complete the data necessary for the comparison of the effects of the metals on the different biological parameters of the crayfish.

MATERIALS AND METHODS

Experimental Design

All studied crayfish were adult intermoult males of the same size and length and were all caught from the same swamp (Vigueirat swamp of Camargue, south of France; GPS coordinates: 43°31.863'N–4°45.417'E). The specimens weighed around 25 ± 2.75 g and were about 9 ± 0.3 cm long. All organisms were acclimatized 3 weeks to experimental conditions (12/12 h light/dark photoperiod, in 17 ± 1°C synthetic water (Al Kaddissi et al., 2011, 2012a, b, c) at pH 6.7 ± 0.2). During the acute exposure tests, groups of 10 animals were exposed up to 10 days to 0 μM of contaminants (control), 40 μM Cd (from $CdCl_2$ stock solution, 0.01M HCl) or 40 μM U (from DU stock solution $UO_2(NO_3)_2$, $6H_2O$, 0.01 M HCl) ($n = 30$) while during the chronic exposure experiment, groups of 10 animals were exposed for a maximum of 60 days to 0 μM of contaminants, 0.1 μM Cd (from $CdCl_2$ stock solution, 0.016 M HNO_3) or 0.1 μM U (from DU stock solution $UO_2(NO_3)_2$, $6H_2O$, 0.016 M HNO_3) ($n = 30$). For each type of exposure (acute/chronic) the contamination of crayfish to U and Cd were conducted simultaneously. This means that all the biological organisms were in the same physiological state were of the same age (size/length), the same stage of the molting cycle, were caught on the same day from the same swamp, acclimatized simultaneously, were fed of the same diet or starved for the same period and the abiotic conditions were similar between studies so only the type of the contaminant varied between conditions of exposure in both experiments. Consequently, the comparison between effects of the different metals on crayfish is reliable. All animals were kept at a density of ~3 crayfish/L and each individual was isolated in a chamber (cylinder made from plastic netting: 1 cm mesh and 11 cm diameter) to avoid injuries due to fights. Mortality of crayfish was checked on a daily basis. The water column was renewed daily in all experiments: 50% of the test solution was changed in the acute experiment while 25% was renewed in the chronic experiment to reduce the volume of waste water solutions at the end of the tests. Cd and U concentrations were measured at least once after renewal and adjusted to ensure mean values close to nominal contamination levels. In the acute exposure experiment, the measured mean concentrations of Cd and U in water were 46.17 ± 6.05 and 35.04 ± 10.5 μM, respectively [$n = 20$, mean ± standard error of the mean (SEM)]. In the chronic exposure experiment, the mean measured concentration of Cd was $0.092 \pm 4.3\ 10^{-3}$ μM while that of U was of $0.12 \pm 4.36\ 10^{-3}$ μM ($n = 60$, mean ± SEM). The temperature was maintained at 17.06 ± 0.23°C and the pH was monitored throughout the exposures (pH acute: 7.01 ± 0.24; pH chronic: 6.22 ± 0.045). The concentrations of the major ions in water (NO_3^-, NO_2^-, SO_4^{2-}, Cl^-) were checked by ion chromatography system (ICS-3000) during the experiments and were always identical to the desired concentrations ($NO_3^- = 76.5$, $NO_2^- < 5\ 10^{-5}$, $SO_4^{2-} = 509$, and $Cl^- = 4100$ μM). At days 4 (T4), 10 (T10), 30 (T30), and 60 (T60), 4 or 5 living crayfish were sampled and sacrificed from each exposure condition. Hepatopancreas and gills were collected from each individual and divided into parts for further biochemical analyses. The parts designated for microscopic analyses were freshly prepared to this end, while others were stored at −80°C until needed.

Metal Quantification

The organs of all experiments were first dried for 2 days at 45°C and then digested in the same manner before analyses of the bioaccumulation (Al Kaddissi et al., 2011, 2012a, b).

Water and biological samples from all experiments were all acidified by nitric acid (3.1 mM) prior to metal quantification. Samples contaminated with U were analyzed for the highest U concentrations by inductively coupled plasma-atomic emission spectrometry (ICP-AES Optima 4300DV, PerkinElmer, Wellesley/USA; detection limit: 10 μg U/L ± 10%) and by inductively coupled plasma-mass spectrometry (ICP-MS Agilent 7500 Cx; quantification limit: 10 $ng.L^{-1}$ ± 7%) for the lowest concentrations. Cd concentrations were measured by atomic absorption spectrophotometry using a graphite furnace (4110 ZL; Perkin-Elmer; detection limit: 0.4 μg Cd/L).

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, then washed three times for 5 min with 0.1 M sodium cacodylate buffer (pH 7.4), and then postfixed with 1% osmium tetroxide in the same buffer for 1 h (Al Kaddissi et al., 2011, 2012b). Samples were subsequently dehydrated with successive baths of ethanol and propylene oxide, and finally embedded in Epon 812 (TAAB). Samples were cut in semithin sections of 200–500 nm for light microscopy analysis. These semithin sections were stained with aqueous blue toluidine then observed under a light microscope (Leica, DM 750) and images were saved using a Leica camera ICC50 and LAS EZ software. Ultrathin sections of 80 and 140 nm 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). All sections were obtained using an ultramicrotome (UCT, Leica). The localization of Cd in the ultrathin sections was conducted using the Energy Dispersive X-ray analyzer equipped with a super ultrathin window model sapphire (EDAX) as like as for DU described previously (Al Kaddissi et al., 2011). 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.

Genetic Analysis

Total RNA were extracted from gills and hepatopancreas in all experiments and then cDNA were synthesized using always the same techniques (Al Kaddissi et al., 2011, 2012a, b). Real-time PCR reactions (1 cycle: 10 min at 95°C, then 50 cycles: 5s at 95°C, 5 s at 60°C, and 20 s at 72°C) using cDNA of samples from all exposure conditions of both experiments were performed in a light cycler (Roche) following the manufacturer's instructions using the DNA intercalating dye SybrGreen I. PCR specificity was determined for each reaction from the dissociation curve of the PCR product which was obtained by following the SybrGreen fluorescence level during a gradual heating from 60–95°C. 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 specific gene and the cycle threshold of the *18S* housekeeping gene (to normalize genes expression levels). Therefore, the expression's factor (EF) of each gene in comparison with control corresponds to the following equation: $EF = 2^{-\Delta CT}(\text{Treatment})/\ 2^{-\Delta CT}(\text{Control})$.

Enzymatic Activities Analysis

Biochemical determinations have been described previously (Al Kaddissi et al., 2012a, b, c). Hereafter, we only provide a brief description. Enzymatic activities were determined in the cellular cytosolic fractions of tissues sampled from the chronic exposure experiment to determine cellular oxidative stress. After homogenization of the tissues, activities of SOD were determined spectrophotometrically using "19160 SOD determination Kit" (Fulka) ($\lambda = 450$ nm). CAT activities were measured by following the decrease in absorbance at 240 nm due to H_2O_2 consumption ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$). GPX activities were assessed by following the oxidation of NaDPH at 340 nm using cumene hydroperoxide as a substrate ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). GST activity was measured with 1-chloro-2.4-dinitrobenzene (CDNB) as substrate. This assay measured the formation of the conjugate with GSH at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). Protein concentration was determined using "Micro BCATM Protein Assay Kit" (Thermo scientific) ($\lambda = 562$ nm). All biochemical analyses were performed with SOFTmax PRO spectrophotometer at a constant temperature of 25°C.

Statistical Analysis

Results are given as mean ± the 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 presented using Kaplan Meier's estimator (Kaplan, 1983). This method estimates the fraction of living organisms for a certain amount of time after treatment and takes into account "censored" data (i.e., losses of crayfish by sampling at T4 before the final outcome is observed at T10). The significant effects of factors (i.e., time and types of metals) on bioaccumulation were performed using analysis of variance, after checking assumptions of normality and variance homogeneity of residuals. When the assumptions were not satisfied, a logarithmic data transformation was applied. If the assumptions were still not satisfied the Kruskal–Wallis nonparametric test was used. To compare the effects of Cd and U on various genes (T4) EF and on antioxidant activities in both organs and at different sampling times for each experiment, principal component analysis (PCA) were necessary to visualize such high-dimensionality and to simplify exploration of the results.

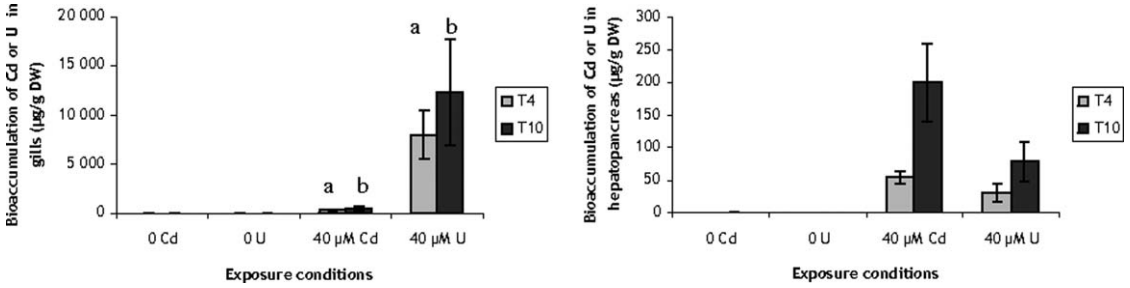

Fig. 1. Concentrations of U and Cd in gills and hepatopancreas of crayfish *P. clarkii* exposed to 40 µM of Cd or U for 4 to 10 (T10) days (µg/g, mean ± SEM, $n = 4$ except for U contaminated organs at T10, where $n = 5$). (a,b) concentrations of U and Cd designated with the same letters are significantly different from each other ($P < 0.05$).

PCAs were performed on a covariance matrix based on the EF and on a correlation matrix based on enzymes activities. The data were arranged in a matrix of n rows and m columns, in which n is the number of conditions (Organ-metals-time) and m the number of variables (genes or enzymes). When the expression levels of certain genes were not different significantly from control, the EF was indicated as equal to 1 in the ACP matrix. The number of principal components (PCs) selected for the presentation of the results was based on Kaiser's rule (Kaiser and Rice, 1974). This criterion retains axes explaining 82% of the total inertia (variance), thus it was possible to restrict the PCA to the first two PCs.

RESULTS

Survival Rate

No mortality was recorded in control groups of both experiments. In groups of crayfish exposed 10 days to 40 µM of Cd, 33% of mortality was observed, while only 10% was detected when exposed to the same concentration of U and period of time. Besides that 100% of the crayfish survived the chronic exposure to Cd and DU.

Bioaccumulation of DU and Cd

Cd and DU accumulations in organs in the acute experiment are shown in Figure 1. Contaminated gills and hepatopancreas were significantly different from controls at T4 and T10 ($P < 0.05$). The bioaccumulation of U and Cd was time dependent only in the hepatopancreas ($P < 0.05$). Both metals were more concentrated in the gills rather than in the hepatopancreas when exposed to high concentrations of the metals. Bioaccumulation of U in gills was about 23 (T4) to 27 (T10) times higher than the bioaccumulation of Cd in the same type of organ ($P \leq 0.05$). In fact, in gills the accumulation factors (AF= metal concentration in organ (µg/g)/metal concentration in water (µg/ml)) of U at T4 (AF = 942) and T10 (AF = 1480) were much higher than those of Cd at the same sampling times (T4: AF = 65; T10: AF = 89). No significant difference was noted between the concentrations of Cd and U in the hepatopancreas in the acute exposure experiment (T4: $P = 0.24$; T10: $P = 0.1$).

Metal accumulation in organs in the chronic experiment is shown in Figure 2. A significant difference was also observed between contaminated organs of crayfish exposed to 0.1 µM of Cd or U and controls ($P < 0.05$). The accumulation of the metals was not time dependent in any organ ($P > 0.05$). Even at lower levels of exposure gills accumulated more U than Cd at T30 (38 times more, $P = 0.00034$) and T60 (22 times more, $P = 0.0013$). The AF of U in gills was up to 14 times higher than that of Cd (ex: $AF_{U\ T30} = 5918$; $AF_{Cd\ T30} = 427$). Moreover, U accumulation levels were higher in gills than in hepatopancreas whereas, the opposite was observed for Cd. In fact, Cd concentrations in hepatopancreas were three times higher than Cd concentrations in

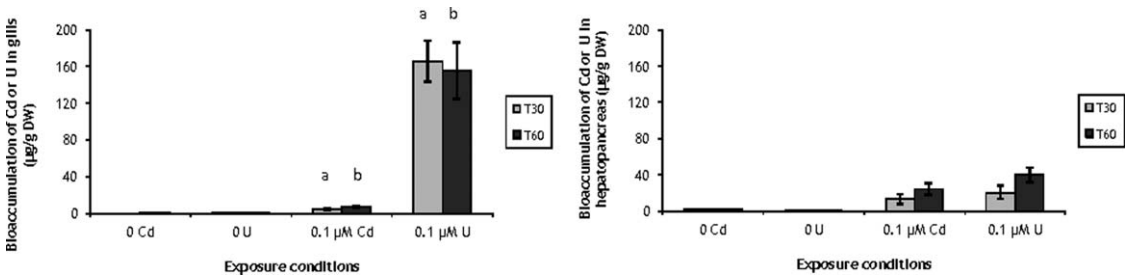

Fig. 2. Concentrations of U and Cd in gills and hepatopancreas of crayfish *P. clarkii* exposed to 0.1 µM of Cd or U for 30 (T30) to 60 (T60) days (µg/g, mean ± SEM, $n = 5$). (a,b) concentrations of U and Cd designated with the same letters are significantly different from each other ($P < 0.05$).

gills at T30 and T60. No significant differences were observed between the bioaccumulation levels of U and Cd in the hepatopancreas in the long term exposure experiment (T30: $P = 0.412$; T60: $P = 0.154$).

Metals Localization and Histological Alterations

Cd was hard to localize by TEM-EDX, however flocculent forms of this contaminant were found in the epithelium and cuticle of gills exposed to 40 μM at T10 (Fig. 3). In the epithelium [Fig. 3(A)], Cd was colocalized mainly with phosphorus, chlorine, and calcium, and to a lesser extent with silicon, aluminium, magnesium, sulfur, and chlorine. In the cuticle [Fig. 3(B)], the contaminant was chiefly associated with phosphorus and to a lesser extent with chlorine and the other elements already cited above. However, U was easily localized in the epithelium of gills under the same conditions of exposure (40 μM; T10). Indeed TEM-EDX analysis of the previous study (Al Kaddissi et al., 2011) led to the detection of U which was colocalized mainly with phosphorus in flake shaped structures.

Vacuolization was detected in some contaminated tubules in the hepatopancreas at the end of the chronic Cd exposure

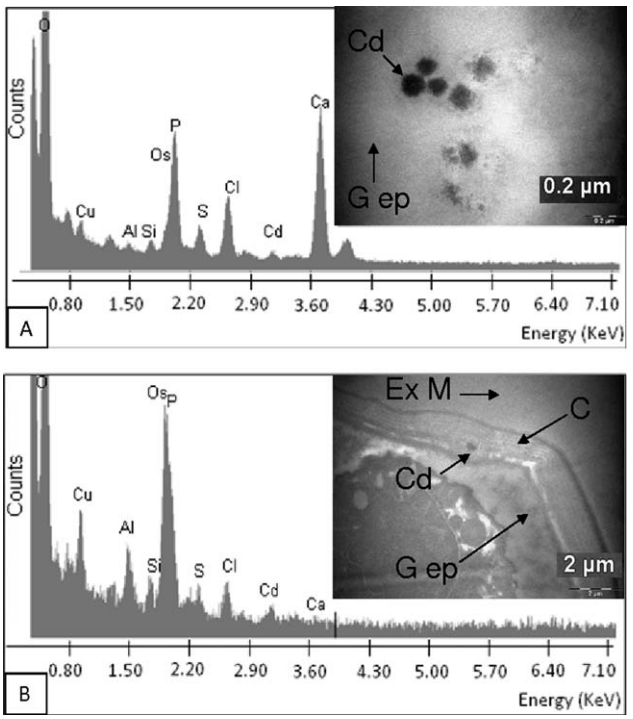

Fig. 3. (A, B): Transmission electron micrographs coupled with energy X-ray results of *P. clarkii* gills exposed for 10 days to 40 μM of Cd. Elements detected in matrix: O: oxygen, Os: osmium, and Cu: copper. Elements detected in the tissues: Cd: cadmium, Al: aluminium, Si: silicon, S: sulfur, Cl: chlorine, and Ca: calcium. (EX M) extracellular matrix, (C) cuticle, and (G ep) gill epithelium.

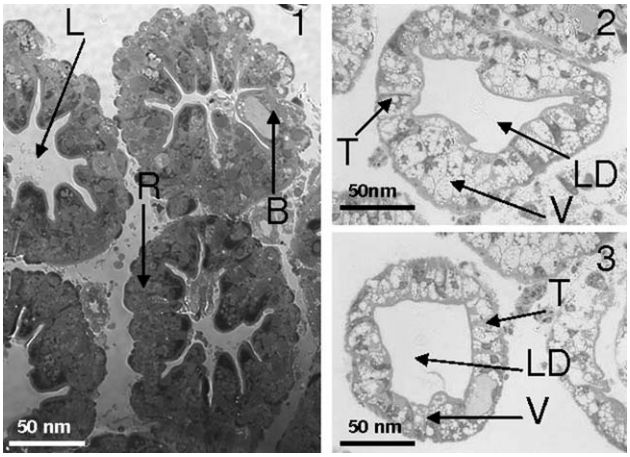

Fig. 4. Histological alterations in *P. clarkii* hepatopancreas tubules after 60 days of exposure to dissolved U: (1) control, (2 and 3) 0.1 μM U. (R) absorptive cells. (B) Secretory cells. (L) Lumen. The pathologies observed were: (T) thinning, (V) vacuolization, and (LD) lumen dilatation.

experiment (0.1 μM; 60 days) in the preceding work (Al Kaddissi et al., 2012b). Here, a distinct impairment of tissue structure was discerned in the hepatopancreas after 60 days of contamination to 0.1 μM of U (Fig. 4). The epithelium of several tubules showed an overall presence of numerous pathologically altered cells with cytoplasmic vacuoles. A lumen dilatation and thinning of the epithelium were also observed, probably due to a loss of epithelial organization. However, this phenomenon was confined to individual tubules and did not affect the entire digestive gland, so some intact glandular epithelium was present even after a long period of contamination.

Enzymatic Responses in Gills Contaminated Chronically

PCA results for gills of crayfish contaminated 30 and 60 days to 0.1μM of Cd or U were characterized by antioxidant activities and presented in Figure 5. Gills which had similar enzymatic activities plotted near one another and formed groups. In this analysis the first PC (PC1) accounted for 51.79% of the variance in the enzymatic responses, while the other PC (PC2) accounted for 30.34%. The variables GPX (28.6%), GST (29.26%), and SOD (30.31%) participated the most in the construction of PC1, whereas CAT contributed 50.75% to the construction of PC2. Control groups especially at T60 were correlated the most to the variables CAT and GST while contaminated gills at T30 seemed mostly negatively correlated to these two variables and positively correlated to GPX and SOD. The distribution of Cd-contaminated gills sampled at T60 was difficult to assess visually. However, the coordinates of these individuals (given by the R program) were closest to the coordinates of CAT and GST. In contrast, U-contaminated organs at T60

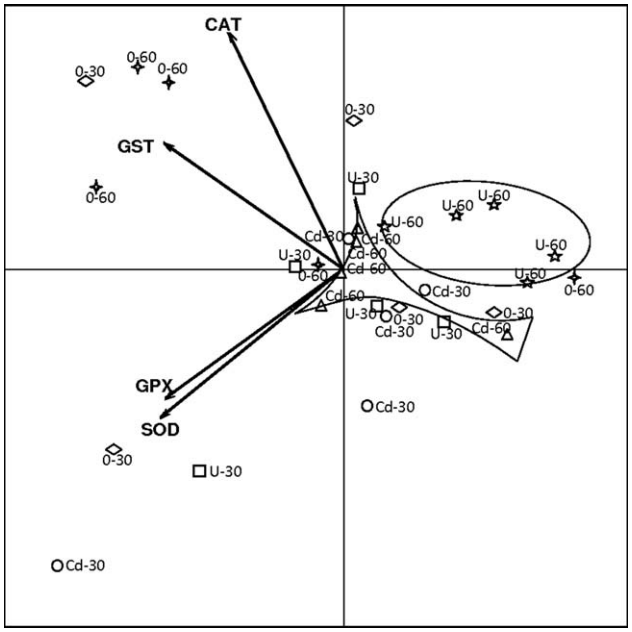

Fig. 5. PCA ordination of gills of crayfish *P. clarkii* exposed to 0.1 μM of U or Cd at T30 and T60 characterized by the activities of SOD (Superoxide dismutase: U/mg Prot), CAT (Catalase: μmol/min/mg Prot), GPX (Glutathione peroxidise: μmol/min/mg Prot), and GST (Glutathione S transferase: μmol/min/mg Prot). Note: clusters of contaminated organs at T60 were manually added for better visualization.

were negatively correlated to all the variables. Enzymatic activities in contaminated hepatopancreas were overall not significantly different from controls and thus PCA results were not presented here.

Expression Factors of the Studied Genes

PCA ordination of the contaminated organs at different sampling times characterized by the EFs of *12s*, *atp6*, *cox1*, *sod(Mn)*, and *mt* are reported in Figure 6 for the acute exposure experiment and in Figure 7 for the chronic exposure experiment. Both ordinations (acute/chronic) arranged hepatopancreas and gills according to transcriptional responses. Organs having similar gene expression levels tended to plot near one another and formed clusters. In the acute exposure experiment, the first PC (PC1) alone accounted for 95.53% of the variance of genes expressions in the organs, whereas the PC2 explained only 3.97% of the variance. The variable *atp6* contributed 99.82% to the construction of PC1 while *mt* contributed the most to the construction of the PC2 (95.93%) followed by *12S* (3.79%). The lengths of the arrows of the vectors (variables) are proportional to the contribution of the variables to the construction of the axes. Thus it is obvious that *cox1*and *sod(Mn)* present the least contribution to the arrangement of the axes (0.19% for PC2 and 0.05% for PC1, respectively). Cd contaminated organs that were sampled at T4 loaded highest on the first axis

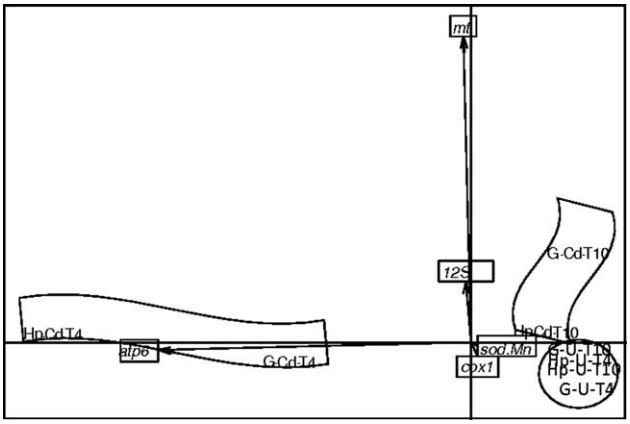

Fig. 6. PCA ordination of hepatopancreas and gills of crayfish *P. clarkii* exposed to 40 μM of U or Cd at T4 and T10 using the EFs of *12s*, *atp6*, *cox1*, *sod(Mn)*, and *mt* compared to the basal level of controls ($n = 5$). Note: clusters were manually added for better visualization.

while the T10- Cd contaminated organs loaded the most on the second axis. Nonetheless, U contaminated organs were negatively correlated to these variables.

In the chronic exposure experiment (Fig. 7) the first PC (PC1) alone accounted for 96.45% of the variance of genes expressions in the organs. The first two components (PC1+PC2) explained together 98.67% of the variance; this means that the loss of information due to the projection of the plots was insignificant. Moreover, the variable *atp6* contributed to 99.26% of the inertia (variance) of the CP1 while the construction of the CP2 was attributed to *12S* (31.18%), *mt* (30.87%), *sod(Mn)* (30.16%), and *cox1* (7.67%). The coordinates of the plot representing Cd-contaminated gills sampled at T30 was strongly correlated to the variable *atp6* while the correlation of Cd-hepatopancreas at T30 with the variables was difficult to assess visually. However, the coordinates of this last plot were obtained by R program and demonstrated that they were closest to the variables *mt*, *12s*, and *atp6*. Cd-contaminated organs sampled at T60 were also

Fig. 7. PCA ordination of hepatopancreas and gills of crayfish *P. clarkii* exposed to 0.1 μM of U or Cd at T30 and T60 characterized by the EFs of *12s*, *atp6*, *cox1*, *sod(Mn)*, and *mt* compared to the basal level of controls ($n = 5$). Note: clusters were manually added for better visualization.

closest to the variable *mt*, *12s* and also to *atp6*. The U-contaminated gills sampled at T30 were significantly negatively correlated to the variable *atp6* while all other U contaminated organs were correlated to the variables *atp6*, *cox1*, and *sod(Mn)*.

DISCUSSION

Comparison of the Mortality Rates

The survival rate of the crayfish *P. clarkii* was lower in groups contaminated to 40 μM of Cd than in groups exposed to the same concentration of DU. Studies comparing the effects of DU and Cd on the survival rates of aquatic invertebrates are scarce. A study on the resistance of freshwater benthic macroinvertebrate *Tubifex tubifex* to U via an exposure to contaminated sediments showed that the LC_{50}-12 days for this species is equal to 2910 $mgUg^{-1}$ dry weight (Lagauzère et al., 2009a, b). Authors compared the sensitivity of this worm with other heavy metals already reported in literature and stated that the toxicity of U to *T. tubifex* seemed lower than copper, Cd, and nickel (Milani et al., 2003). Martins et al. (2007) summarized in their review the Cd LC_{50}-48h values of the microcrustacean *D. magna* found in literature. The lowest concentration reported was equal to 0.009 mg $Cd.L^{-1}$(Lewis and Horning Ii, 1991) whereas the highest was of 0.065 mg Cd/L (Toussaint et al., 1995). While the lowest U LC_{50}-48h for *D. magna* reported by Zeeman et al. (2008) was equal to 0.39 mg U/L while the highest was of 7.8 mg U/L. These findings show that *D. magna* seemed more tolerant to U toxicity than Cd. Moreover, the Cd LC_{50}-96h of the fish *Danio rerio* is around 2.2 mg Cd/L (Martins et al., 2007), where the U LC_{50}-96h for the same species is almost of the same range and equal to 3.05 mg U/L (Labrot et al., 1999). However, the different conditions under which the assays on the fish were performed (pH, water hardness, temperature. . .) make it difficult to draw any relevant comparison. Moreover, the crayfish mortality rates in this study were low demonstrating the resistance capacities of *P. clarkii*. This crayfish also resisted long term exposure (60 days) to 0.1 μM of Cd and U. Wigginton and Birge (2007) demonstrated in the same study that *P. clarkii* was more resistant to Cd exposure when compared with other crayfish species (*Orconectes* sp. and *Procambarus* sp.). In addition, this species tolerates high concentrations of metals of greatest toxicological significance, such as lead (Pb) and mercury (Hg) (Del Ramo et al., 1987; Torreblanca et al., 1989; Naqvi and Howell, 1993). In fact *P. clarkii* has been used as a bioindicator of trace metal toxicity in numerous field studies (Finerty et al., 1990; Sánchez López et al., 2004; Higueras et al., 2006; Hothem et al., 2007; Richert and Sneddon, 2008; Faria et al., 2010; Moss et al., 2010; Suárez-Serrano et al., 2010). Therefore, the tolerance of this species toward U and Cd make these organisms good candidates to be used as alert systems in case of acute water contamination by these two metals, such as accidental spills, industrial wastewater discharge, and accidental or intended water poisoning.

Comparison of the Bioaccumulation and Detoxification

This species seemed to be more resistant to U despite of its high levels of concentrations in organs after the acute exposure. The crayfish bioaccumulated 23–27 times more U in the gills than Cd. A possible explanation could be differences in the detoxification mechanisms between both metals. The electron probe X-ray microanalysis detected Cd in flocculent forms which were localized in the epithelium and cuticle of gills exposed to 40 μM of Cd at T10. Deposits of Cd were observed by Tayal et al. (2000) in fish *Colisa fasciatus* cells, where they were visualized as dense clouds. They demonstrated that Cd in presence of Na_2S is converted into insoluble metal sulfides. In our experiment, Cd was colocalized mainly with phosphorus, chlorine and calcium. A similar description was reported in cells of *Azolla filiculoides* shoots. The analysis of Cd content in the shoot demonstrated the appearance of dark grains about 0.3 μm, the Cd in these grains was accompanied by phosphorus and calcium (Sela et al., 1988). Indeed, heavy metal containing granules found in epithelial cells of invertebrates may contain either calcium or heavy metals, such as zinc, copper, iron, and so forth, complexed with sulfur or phosphorus (Ahearn et al., 2004). As a result of these complexation events with anions, the heavy-metal ion is removed from the cytoplasm by sequestration within a vacuolar membrane in an insoluble, detoxified form. Subsequent cellular exocytotic events may extrude the concretions from the cell followed by excretory mechanisms (Ahearn et al., 2004; Marigómez et al., 2002). Moreover, TEM-EDX analysis led to the detection of piles of flake shaped U inside the gills epithelium after 10 days of exposure to 40 μM of DU (Al Kaddissi et al., 2011). This form of U was mainly colocalized with phosphorus which is insoluble and nontoxic. Similar results were also observed in the bivalve *Corbicula fluminea* (Simon et al., 2011). It should be noted that Cd was harder to localize by TEM-EDX than U as it could have strongly bound to organic ligands, such as proteins (e.g., metallothioneins (MT)), lipids, and nucleic acids. In these cases, it may not have been complexed with sulfur or phosphorus and therefore, remained histochemically invisible. Thus once the metals are absorbed, it is possible that Cd binds to organic ligands quicker than U. Another explanation to the higher levels of U detected in gills when compared with Cd could be related to differences in the adsorption capacities of the metals on tissues. Choi et al. (2009) demonstrated that the adsorption capacity of Cd, U, and Pb on starfish and bacterial cells followed the order: U(VI)>Pb>Cd. It seems that cells preferentially sorb U, followed by Pb, and exhibit the least preference for Cd. It was suggested that the mechanisms for metal uptake process are

mainly both organism- and metal-dependent because of specific surface properties of the organisms, cell physiology, and solution chemistry. In the chronic experiment gills also accumulated more U than Cd possibly for the same reasons stated above. Recently, Simon et al. (2013) demonstrated that only 12% of total U accumulation in gills of crayfish O*rconectes limosus* after waterborne exposure was measured in the cytosol fraction which is considered as the potential toxic fraction. Authors stated that adsorption of U on the gills cuticle of *O. limosus* represented the main contribution of U accumulation. These findings point out the importance of identifying the metal bioavailable fraction in future studies. Interestingly, when comparing the bioaccumulation levels between organs different results were obtained for the acute and chronic experiments in the case of Cd. In fact, Cd concentrations were lower in gills than in hepatopancreas when contaminated chronically, while the opposite was observed in acute exposure conditions. This could be related to differences in adsorption rates on tissues of the different waterborne Cd concentrations (40 μM vs. 0.1 μM) (Al Kaddissi et al., 2012b). In U exposure conditions, gills always bioaccumulated more U when compared with the hepatopancreas no matter the type of exposure (acute/chronic). These results also demonstrate that Cd and U have different transfer capacities to organs. Indeed, in the acute exposure experiment crayfish accumulated up to 263 times more U in gills than in hepatopancreas whereas this factor was maximum equal to 6 for Cd. This shows that Cd is more easily transferred to organs than U. This hypothesis is also verified in the chronic exposure experiment since the hepatopancreas bioaccumulated more Cd than gills and the opposite was observed for U. Examination of the storage and distribution of Cd and U in roots and shoots of the plant *Azolla filiculoides* showed that Cd relative content in the shoot was similar to its content in the root, hence its mobility was relatively high. Authors also showed that the absence of significant U quantities in the shoot despite of the relatively high content in the root suggest a lesser mobility of U than Cd from *A. filiculoides* root (Sela et al., 1988). These findings are in concordance with our results. A tendency of increasing metal bioaccumulation with time was observed in organs sampled from the acute exposure experiment even though statistically it was only significant in hepatopancreas. Then again, the chronic Cd and U bioaccumulation in gills seemed to have reached a steady state at T30 and T60 on the opposite to the accumulation in the hepatopancreas. At last, crayfish were able to bioconcentrate rapidly both contaminants in their tissues after exposition to 40 μM (high AF at T4: e.g., AFgills-40 μM Cd$\sim$ 65 and AF gills-40 μMU$\sim$ 942). *P. clarkii* was found to be also capable of concentrating U after 4 days in gills and hepatopancreas even after a low level of waterborne exposure (0.1 μM U) (Al Kaddissi et al., 2011, 2012c). Diaz-Mayans et al. (1986), also demonstrated that *P. clarkii* can bioaccumulate Cd in these tissues after 4 days when exposed to 0.1 μM *via* a direct route of exposure. This capacity of the crayfish to bioaccumulate the studied metals early can be useful in field studies, caging experiments and biomonitoring programs.

Comparison of Histopathological Effects

A previous study has shown that U induces histological damages to the hepatopancreas of *P. clarkii* after 10 days of exposure to 40 μM of U (Al Kaddissi et al., 2011). In this study, we demonstrated that this metal provoked similar damage after a long term exposure (60 days) to a low concentration of waterborne U (0.1 μM). In fact, the histopathology was identical; both experiments had demonstrated that U causes vacuolization, lumen dilatation, and thinning of the epithelium of the hepatopancreas. However, in the U acute exposure experiment we have additionally examined a disorganization of the epithelium and evidenced the presence of degenerated tubules. This shows that the extent of histopathological damage was in line with the bioaccumulation of U determined in the soft tissues in both experiments. The concentration of U recorded in the hepatopancreas after 10 days of exposure to 40 μM (78.8 $\pm$ 31.2 μg U/ g DW) was higher than the concentration found after 60 days of exposure to 0.1 μM (39.91 $\pm$ 8.07 μg U/ g DW). In a previous study, we have demonstrated that the epithelium of the hepatopancreas of *P. clarkii* showed vacuolization after 60 days of exposure to 0.1 μM of Cd (Al Kaddissi et al., 2012b). This demonstrates that vacuolization is a similar symptom of both Cd and U toxicity. Moreover, vacuolization in hepatopancreatic cells of the green mussel *Perna viridis* exposed to various concentrations of copper and mercury were observed in various degrees (Pillai and Menon, 1998). Additionally, one of the prominent effects of platinum on the liver of the fish *Danio rerio* and on the hepatopancreas of the ramshorn snail *Marisa cornuarietis* is increased vacuolization in cells (Osterauer et al., 2010). Exposure of the crayfish *P. clarkii* to high levels of Cd concentrations also revealed structural damage in the hepatopancreas. For example, when exposed to 40 μM Cd for 10 days vacuolization, lumen dilatation, thinning of the epithelium, and cellular shrinkage were observed in this organ (Al Kaddissi et al., 2012b). It should be highlighted that these symptoms were identical to those observed in the hepatopancreas of *P. clarkii* exposed to 40 μM of DU indicating that they are not metal specific. Moreover degenerated tubules were seen in these organs which indicate that DU can lead to cell death. In fact studies have already reported that U can induce apoptosis and necrosis in cells (Thiébault et al., 2007; Periyakaruppan et al., 2009). It is also known that cellular shrinkage is the first sign of programmed cell death (Rost-Roszkowska et al., 2010), thus we suspect that 40 μM of Cd led to apoptosis of hepatopancreatic tubules in crayfish. In fact, it has already been confirmed that apoptosis is one of the hepatopancreas cell death pathways of the crab *Sinopotamon yangtsekiense* on Cd administration (Li et al., 2010). Cd is also capable of provoking

necrosis in cells, indeed histopathological examination showed tissue injuries consistent with inflammation and necrosis in the digestive gland of the common mussel *Mytilus edulis* during Cd exposure (11 days at 20 and 50 μg/L) (Sheir and Handy, 2010). At this level of comparison it is difficult to draw conclusions on the classification of the cytotoxic effects of U and Cd because no statistical analyses were conducted to quantify these impacts. However, a comparative study between the cytotoxicity of U and other metals on the osteoblastic bone cells of rats was conducted and permitted to classify Cd, Zn, Se^{IV}, and Cu as the most toxic and Ni, Se^{VI}, Mn, and U as the least toxic. The toxicity of metals was determined from the quantification of cell viability by comparison to controls (Milgram et al., 2007).

Comparison of Antioxidant Responses

Exposed gills of *P. clarkii* sampled after the chronic experiment with similar enzymatic activities were grouped together (Fig. 5). The enzymatic responses in contaminated gills of *P. clarkii* seemed influenced by exposure time since the organs at T60 were better separated from their corresponded control than at T30 indicating a more important oxidative stress at T60 than T30. At T30 the contaminated gills (especially to U) were close to the corresponding control organs and formed a cluster demonstrating that the activities of the enzymes were not significantly different in these conditions. Two other distinctive clusters were also formed, the first contained the organs contaminated to U during 60 days and the second consisted of the contaminated gills with Cd at T60. This ordination of the plots demonstrated the differences between the effects of Cd and U on this level of biological organization. The contaminated organs with Cd and U at T60 were oppositely detached from their corresponding control group and thus were all negatively correlated to CAT and GST. However, contaminated gills with U at T60 were more isolated from the control groups and from all the other conditions and were negatively correlated to all the enzymes. This result indicated that the enzymatic responses of all the studied antioxidants tended to decrease at the end of the experiment in presence of U. The decrease in antioxidants responses in presence of Cd or U could be linked to a decrease in substrates, expressions levels of the corresponding genes, oxidative alteration of the enzymes, and/or direct binding of the contaminants to the proteins (Al Kaddissi et al., 2012a, b). In fact, Bem and Bou-Rabee (2004) stated that the chemical toxicity of uranium should be similar to that for other heavy metals, for example, cadmium or mercury because of its chemical affinity to proteins and formation of stable complexes with biological ligands of low molecular mass. GPX ($P = 0.0054$) and SOD ($P = 0.037$) activities were significantly more depleted in U contaminated organs than in those exposed to Cd at T60. Therefore, it seemed that the oxidative stress was more important in the U exposed organs which should be in concordance with the bioaccumulation levels. Even though U concentration in gills was 22 times higher than that of Cd, the observed decrease in enzyme activities was low when compared with the Cd contaminated condition (GPX: $-33.15\ \% \pm 3.24$, GST: $-6.3\ \% \pm 6.5$, CAT: $-24\ \% \pm 20$, and SOD: $-18.6\% \pm 7.06$). It should be reminded that the bioaccumulation levels result from adsorption, internalization of the metal, and the detoxification mechanisms. Thus it is possible that a part of U was precipitated in a detoxified form and/or adsorbed on the cuticle and remained biologically unavailable. Consequently, it is not possible to indicate whether or not Cd is capable to generate more oxidative stress than U, nor to classify the toxicity of the metals at this level of biological organization. Moreover, the mechanisms by which U and Cd generate oxidative stress are different which complicate the comparison between the toxicity of both metals. Indeed, it is well known that Cd promotes indirectly oxidative stress in cells (Wang et al., 2004; Shi et al., 2005) because it is a redox-inactive metal that cannot induce fenton-like reactions (Ercal et al., 2001). Nonetheless, U 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). Furthermore, U can enhance the production of free radicals via the ionization phenomenon induced by alpha particle emissions (Jones et al., 2003).

Comparison of the Variations in Transcriptional Responses

It has already been proven that both Cd and U impact the mitochondria (Pulido and Parrish, 2003; Valko et al., 2005; Pourahmad et al., 2006; Cannino et al., 2009; Lerebours et al., 2009, 2010; Al Kaddissi et al., 2011, 2012a, b) and generate oxidative stress (Stohs et al., 2001; Gonzalez et al., 2006; Barillet et al., 2007; Jia et al., 2011; Wang et al., 2010; Barillet et al., 2011; Al Kaddissi et al., 2012a, b). In previous studies, we evidenced alterations in the expression levels of mitochondrial genes (*cox1, atp6*, and *12S*) and genes involved in the oxidative stress responses (*sod(Mn)* and *mt*) in gills and hepatopancreas of *P. clarkii* exposed to 40 or 0.1 μM of either Cd or U. In this study, we observed that gills and hepatopancreas of crayfish sampled from the acute experiment were separated into groups by PCA using the EFs of *cox1*, *atp6*, *12S*, *sod(Mn)*, and *mt*. All U contaminated organs constituted a tight cluster that was distinctive from the two other groups formed by Cd contaminated organs. In addition, organs sampled at T4 from the Cd exposure conditions were clearly separated from those sampled at T10. The expression levels of *atp6* conditioned the most this organization. Indeed, the most variability in the EFs was observed for the *atp6* gene. In fact, Cd contaminated organs at T4 were associated the most with the variable *atp6* which discriminated this group from all others. Whereas, the *atp6* gene was only threefold up regulated in gills exposed 10

days to 40 μM DU (Al Kaddissi et al., 2011). Toxic metals can have two types of effects: (1) indirect, due to the additional metabolic cost of accumulating, transporting, storing, and excreting the contaminant; and (2) direct, on cellular membranes and/or specific biochemical pathways (Labrot et al., 1999). Without a doubt, the ATP synthase is responsible for both ATP synthesis and hydrolysis (Li and Neufeld, 2001), thus high variations in *atp6* gene expression levels are expected when cells are facing a stress caused by metals. Cells could produce more ATP for maintaining the viability of crayfish and provide additional energy to defense mechanisms (e.g.: transporting, storing, excreting, detoxification. . .), or in certain pathologies the ATP synthase can hydrolyze the ATP as a response to a mitochondrial stress to maintain the H^+ gradient (Nevière, 2008; Wang and Oster, 1998). Either way, the solicitation of the ATP synthase was clearly elevated in contaminated organs. Moreover, Muhlia-Almazan et al. (2008) stated that the level of expression of this gene in the shrimp *Litopenaeus vannamei* can change in response to environmental changes and to endogenous physiological conditions, which makes the expression of this gene predisposed to be highly altered. At T10 Cd contaminated organs were associated the most with the vectors *mt* and *12s* which means that the expression levels of these two genes distinguished the most this group from the others. In fact, *mt* gene was always up-regulated in Cd contaminated organs and was the most expressed in gills at T10 (gills: 39-fold; hepatopancreas: 11-fold) (Al Kaddissi et al., 2012b), while it was only over-expressed in gills contaminated to U at T10 (fivefold up) (Al Kaddissi et al., 2011). Indeed, Cd (Duncan et al., 2006) and U (Michon et al., 2010) can bind to MT. It is also known that the concentration of metals ions required for the induction of MT and the time required for response varies according to the metal (Freedman et al., 1993; Haq et al., 2003). The *mt* gene can also be up-regulated because of an increase in oxidative stress (Iszard et al., 1995; Andrews, 2000; Haq et al., 2003).The *12S* gene expression increased over time in presence of high levels of Cd (Al Kaddissi et al., 2012b), whereas this gene was down-regulated in U contaminated organs at the end of the experiment (Al Kaddissi et al., 2011) indicating a different effect. Even though the variable *Sod(Mn)* and *Cox1* did not contributed the most in the distribution of the groups, it should be mentioned that mostly the *cox1* and *Sod(Mn)* genes were repressed in U and Cd contaminated organs (Al Kaddissi et al., 2011, 2012b). It appears that the pattern of expression of *atp6* and *cox1* genes was similar when organs are exposed to high levels of Cd or U (ex: gills at T10).

In the chronic exposure experiment, distinctive groups were also formed between Cd and U exposed organs (Fig. 7). In fact, Cd contaminated gills and hepatopancreas sampled at T30 were separated from those sampled at T60, while U contaminated organs formed a distinct cluster except for gills sampled at T30 which were clearly separated from all others. The separation of the groups contaminated with Cd from those contaminated with U was especially due to the variations in the expression levels of the genes *atp6*, *mt*, and *12S* as it was observed in the acute exposure experiment. Cd contaminated organs sampled at T60 were better correlated to these variables than those sampled from the Cd exposure condition at T30, which demonstrates higher values in the EFs of these genes at T60. The gills exposed to U and sampled at T30 showed the highest variation in the EF of the *atp6* gene since they were significantly negatively correlated to this variable. The other U contaminated organs seemed to have a similar pattern of gene expression especially for *atp6*, *cox1*, and *sod(Mn)*. However a negative correlation to the variables *cox1* and *sod(Mn)* was observed for the Cd contaminated organs which demonstrates a difference in the pattern of these gene expressions in organs exposed to low levels of Cd or U.

CONCLUSION

To sum up, crayfish *P. clarkii* presented high resistance capacities to both studied contaminants. However, U seemed to be less toxic than Cd to this species since it tolerated U more than Cd despite of the presence of higher levels of U concentrations in organs. This was in part due to differences in detoxification mechanisms. Indeed, we have evidenced the presence of flocculent forms of Cd localized mainly with phosphorus, chlorine and calcium in the epithelium and the cuticle of gills. Whereas, piles of flake shaped U localized mainly with phosphorus was observed in the epithelium of gills. The latter detoxified form was more easily detected than the form of Cd indicating a better detoxification mechanism. We also, pointed out the importance of identifying the metal bioavailable fraction in future studies because U might have more adsorbed on organs than Cd. Crayfish bioaccumulated both contaminants quickly in organs, however, Cd was more easily transferred from gills to the hepatopancreas. This was followed by histopathological damages in the hepatopancreas which were similar for both Cd and U (vacuolization, lumen dilatation, thinning of the epithelium, degenerated tubules. . .). Moreover, it was obvious that both toxic metals impaired the mitochondrial chain reaction and generated oxidative stress. The results of PCA performed on the genes expressions highlighted the large difference in the levels of expression of *atp6, mt*, and *12S* in highly exposed organs to Cd and U. However, these important variations masked the fact that the pattern of expression of all studied genes except for *12S* was identical in gills contaminated 10 days to 40 μM of Cd and U. The later shows that the mechanisms by which Cd and U induce their toxicity at high levels of contamination seemed similar. Contrary to this, the mechanisms by which U and Cd induce oxidative stress and mitochondrial alterations were different at low levels of exposure (chronic exposure). Indeed, the expression pattern of the

studied genes was mostly opposed when compared between Cd and U contaminated conditions. Nevertheless, it is easier to classify metals according to their toxicity by comparing the effects on higher biological levels while a "Top-down" approach (population to molecular levels) has a more mechanistic value (Snape et al., 2004).

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