Effects and bioaccumulation of Cr(III), Cr(VI) and their mixture in the freshwater mussel *Corbicula fluminea*

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1 **Effects and bioaccumulation of Cr(III), Cr(VI) and their mixture in the freshwater mussel**

2 ***Corbicula fluminea***

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Abstract

Chromium has two main oxidation states, Cr(III) and Cr(VI), that can occur simultaneously in natural waters. Current consensus holds that Cr(VI) is of high ecotoxicological concern, but regards Cr(III) as poorly bioavailable and relatively non-toxic. In this work, the effects and bioaccumulation of Cr(III), Cr(VI) and their mixture were studied using the freshwater clam *Corbicula fluminea* as a model organism. Mixture exposures were carried out using solutions isotopically enriched in $^{50}\text{Cr(III)}$ or $^{53}\text{Cr(VI)}$, allowing to quantify the contribution of each redox form to total Cr accumulation in the clams. Following exposure to individual redox forms, Cr(III) accumulated preferentially in the digestive glands and Cr(VI) in the gills of *C. fluminea*. In mixture exposures, both redox forms accumulated mainly in the gills; the concentration of Cr(III) in the digestive glands being much lowered compared with individual exposures. Both oxidation states affected the expression of biomarkers related to energy reserves, cellular damage and mitochondrial functioning, as well as the expression of mRNA for detoxification genes. The observed effects differed between gills and digestive glands. The present study suggests that Cr(III) is a bioavailable and biologically active elemental species deserving more consideration by the ecotoxicological community.

Keywords: chromium speciation, speciation, mixture exposure, stable isotopes, regression analysis, biomarkers

Highlights

Biomarkers expression responds to Cr redox state, concentration and exposure duration

First use of isotopically enriched solutions to study Cr(III)/Cr(VI) mixtures

Both Cr(VI) and Cr(III) accumulate in gills and digestive glands of *Corbicula fluminea*

The presence of Cr(VI) changes the accumulation pattern of Cr(III)

Cr(III) is a biologically active species along with Cr(VI)

1. Introduction

Chromium is a widespread environmental contaminant due to its use in several industrial processes (WHO, 2009; 2013). In natural environments, Cr mostly occurs in two oxidation states, Cr(III) and Cr(VI), that have markedly different environmental and biological behaviour. Chromium(VI) is a recognized human carcinogen and has a high environmental mobility, bioavailability and (eco)toxicity. These biogeochemical properties follow from chromate ions being the most common chemical species of Cr(VI) (Gorny et al., 2016). Chromate ions are structural analogues of sulphates and can easily cross biological membranes via facilitated transport (Viti et al., 2014). Once inside the cell, Cr(VI) undergoes rapid reduction to Cr(III). The reduction reactions cause oxidative stress damage and produce intracellular Cr(III) that can further react with nucleic acids causing mutagenic and genotoxic effects (Kortenkamp et al., 1991; Sawicka et al., 2021; Zhitkovich, 2005). Chromate ions also show limited reactivity towards environmental particles, which usually bear a net negative charge at pH values above 4 (Warren and Haack, 2001). Chromate ions can therefore undergo long-distance transport and migrate into groundwater (Guo et al., 2020; Izbicki et al., 2015; Oze et al., 2007).

Chromium(III) is putatively less bioavailable and (eco)toxic, but increasing evidence suggests that it can also elicit adverse effects in humans as well as in aquatic and terrestrial organisms (Aharchaou et al., 2018; Park, 2020; Tsuchiyama et al., 2020). Mechanism of Cr(III) uptake are less studied than those for Cr(VI) and may involve Cr(III)-transferrin complexes, endocytosis of Cr(III)-bearing particles or uptake of hydrophobic Cr(III) complexes (Beyersmann and Hartwig, 2008; Viti et al., 2014). Effects of Cr(III) have also been reported on the structure and permeability of biological membranes (Filice et al., 2019; Suwalsky et al., 2008). Finally, Cr(III) is particle reactive and tends to adsorb onto suspended particulate matter and colloids eventually accumulating in bed sediments (Dominik et al., 2007; Gustafsson et al., 2014; Liao et al., 2020).

Although ecotoxicological studies are biased toward Cr(VI), risk assessment has been performed for both oxidation states (WHO, 2009; 2013). The World Health Organization (WHO) proposes guideline values (Probable No Effect Concentrations – PNEC) of $10 \mu\text{g L}^{-1}$ for Cr(III) and $4 \mu\text{g L}^{-1}$ for Cr(VI). The Joint Research Centre of the European Commission proposed a PNEC of $1.8 \mu\text{g L}^{-1}$ for Cr(III) based on the result of a chronic test with the teleost zebrafish *Danio rerio* (Gomez Cortes et al., 2020). Several studies also confirmed that both Cr(III) and Cr(VI) can accumulate in marine and freshwater organisms via aqueous and dietary exposure routes (Aharchaou et al., 2017; Baumann and Fisher, 2011; Roditi and Fisher, 1999; Semeniuk et al., 2016; Wang et al., 1997). The net bioaccumulation and biological effects of Cr in real field conditions therefore likely result from the combined uptake of Cr(III) and Cr(VI). The two oxidation states actually occur simultaneously, albeit in variable proportions, in natural field conditions (Ferrari et al., 2019; Gunkel-Grillon et al., 2014; McClain and Maher, 2016; Saputro et al., 2014). To the best of our knowledge, no existing laboratory study has yet examined the effects and bioavailability of Cr during such conditions of simultaneous exposure to Cr(III) and Cr(VI) that organisms often encounter in the field.

In principle, it is relatively easy to prepare laboratory exposure media containing both Cr redox forms. On the other hand, it remains far from straightforward to understand the relative contribution of Cr(III) and Cr(VI) to the total Cr body burden and biological responses of the exposed organisms. In aqueous matrices, including ecotoxicological test media, the redox speciation of Cr can be determined using ion Chromatography ICP-MS (Aharchaou et al., 2018; Markiewicz et al., 2015; Seby et al., 2003). However, the redox speciation of Cr in the exposure medium will not be retained in the test organisms, because Cr(VI) is rapidly reduced to Cr(III) in the intracellular environment (Beyersmann and Hartwig, 2008; Sawicka et al., 2021; Zhitkovich, 2005). Tracking the fate of Cr(III) and Cr(VI) from exposure solution to biological tissues is

however possible using speciated, isotopically enriched solutions (IES). Speciated isotope dilution mass spectrometry has already been used to obtain information on the relative abundance of Cr(III) and Cr(VI) in solid matrices (Martone et al., 2013; Séby and Vacchina, 2018; Unceta et al., 2010). Isotopically enriched solutions have also been used successfully to document the importance of dietary uptake of Cu and Zn by freshwater snails (Croteau et al., 2013; 2017), but similar studies on Cr are still lacking.

Chromium has four naturally occurring stable isotopes ($^{50}\text{Cr} = 4.345\%$, $^{52}\text{Cr} = 83.789\%$, $^{53}\text{Cr} = 9.501\%$ and $^{54}\text{Cr} = 2.365\%$) that are widely studied in relation to cosmochemical, geochemical and environmental processes (Cole et al., 2017; Rodríguez-González et al., 2005; Wei et al., 2020). Ecotoxicological studies on Cr ecotoxicity usually employ commercially available Cr(III) and Cr(VI) salts that contain all four isotopes at the corresponding natural abundances. However, materials enriched in one specific isotope and in a specific redox form are also available for purchase (Martone et al., 2013; Novotnik et al., 2012). In the absence of redox interconversions between Cr(III) and Cr(VI) in the exposure medium, the fate of each isotope (i.e., redox form) can be followed from the exposure solution to the biological tissue via a simple ICP-MS analysis at the right m/z value. In the present study, specimen of the freshwater bivalve *Corbicula fluminea* were exposed to solutions amended with Cr(III) or Cr(VI) salts having natural isotopic abundances, or with a mixture of $^{50}\text{Cr(III)} + ^{53}\text{Cr(VI)}$. *Corbicula fluminea* is a widespread filter-feeder that is largely used as a sentinel organism for freshwater biomonitoring of metal accumulation and sublethal effects (de Oliveira and dos Reis Martinez, 2014; Marie et al., 2006; Pereto et al., 2020). The specific objectives of the present study were to determine: (1) the effect of the individual Cr redox forms and of the $^{50}\text{Cr(III)} + ^{53}\text{Cr(VI)}$ mixture on the expression of several biomarkers and mRNA, and (2) the bioaccumulation of Cr in the bivalves' gills and digestive glands.

2. Materials and methods

2.1. Organism collection and acclimation

Individuals of *Corbicula fluminea* (size 2–4 cm) were collected in the Moselle River (France) at GPS coordinates 49°07'06.5'' N, 6°09'39.0'' E and kept until the end of sampling in unfiltered site water (pH = 8.0, conductivity = 1823 $\mu\text{S cm}^{-1}$, oxygen saturation = 103%). Upon return to the laboratory, organisms were kept in aerated water at 15 ± 0.1 °C and acclimated to the test medium (Cristaline® mineral water, spring Aurèle, see composition in Table S1) by replacing 1/3 site water with spring water at 24 h intervals over four days (Potet et al., 2016). The viability of organisms was checked at each medium renewal by testing their ability to close the valves after a gentle pressure on the ligament.

2.2. Exposure set-up

A preliminary test was performed to establish suitable exposure conditions for the measurement of sublethal effects and bioaccumulation of Cr. After acclimation (section 2.1), twelve individuals of *C. fluminea* were transferred to 3 L aquaria containing 1.5 L of Cristaline® mineral water amended with 5, 50, 500, 1000, 2000 and 5000 $\mu\text{g L}^{-1}$ of Cr(III) or Cr(VI). Trivalent Cr was added as $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (Alfa Aesar, purity 99.5 % min, crystalline, CAS 10060-12-5) and hexavalent Cr as $\text{K}_2\text{Cr}_2\text{O}_7$ (VWR, purity 99.8 % min, CAS 7778-50-9). Both Cr salts had natural isotopic abundances (NIA). Exposure medium was continuously aerated by bubbling ambient air in each aquarium. Organisms were kept at 15 °C under artificial daylight (800–1000 Lux) and a 12 h light – 12 h dark photoperiod. Organisms were not fed during the experiments. No mortality was observed across the entire concentration range for neither Cr(III) nor Cr(VI) (data not

shown) and exposure concentrations for subsequent experiments were selected as described in the next paragraph.

In a first experiment (exp #1) for biomarker and mRNA measurements in the gills and digestive glands, organisms were exposed to 5, 50 and 500 $\mu\text{g L}^{-1}$ of either Cr(III) or Cr(VI) using salts with NIA. Exposures lasted for 24 h or 96 h with daily renewal of exposure medium. Chromium concentrations were selected according to three criteria: absence of mortality, potential for sublethal effects and prospective environmental relevance. Twelve acclimated organisms were used for each concentration and exposure time. Each Cr redox form and exposure time were matched to a batch of control organisms not exposed to Cr. Other experimental conditions were the same as previously described.

A second experiment (exp #2) examined the relationships between biomarker expression and Cr bioaccumulation in the gills and digestive glands of *C. fluminea*. Organisms were exposed to: 50 $\mu\text{g L}^{-1}$ of Cr(III) with NIA, 50 $\mu\text{g L}^{-1}$ of Cr(VI) with NIA, or a mixture of 50 $\mu\text{g L}^{-1}$ of Cr(VI) + 50 $\mu\text{g L}^{-1}$ of Cr(III) using IES of $^{53}\text{Cr(VI)}$ and $^{50}\text{Cr(III)}$. Both IES were obtained from Applied Isotope Technologies – AIT, Pittsburg, PA, USA: batch # SH1332, Lot # CR12222014JC; PN-20529, isotopic enrichment 97.7 % for $^{53}\text{Cr(VI)}$ and batch # 030129-04-A, Lot # Cr04272010A; PN-2; isotopic enrichment 97.3 % for $^{50}\text{Cr(III)}$. All tests lasted for 96 h and were carried out under the same conditions as described above with 12 individuals for each scenario plus two control batches of 12 individuals each. The selected concentrations were considered a good compromise between practical constraints (notably the availability of IES and analytical detection in biological tissues) and environmental relevance for contaminated environments (Gunkel-Grillon et al., 2014; Hayzoun et al., 2015).

2.3. Chromium measurements in aqueous and biological matrices

In exp #1, total and total filterable Cr concentrations were measured at $t = 0, 2, 4, 6$ and 24 h ($n = 1$ to 3). Filtration was carried out using $0.45 \mu\text{m}$ membrane filters (Merck Millipore, Millex 33mm, PVDF, reference # SLHV033NB). For experiments lasting 96 h, measurements were carried out over a single renewal period of 24 h and considered representative for all exposure periods. Samples were acidified at 1% v/v with concentrated nitric acid (65% , VWR, Suprapur) and analysed by Flame Atomic Absorption Spectroscopy (FAAS; Perkin-Elmer AAnalyst 100) or Graphite Furnace Flame Atomic Absorption (GFAAS; Varian spectra 880Z+). Measurement accuracy was verified using certified reference materials SPS-SW1 ($2.00 \pm 0.02 \mu\text{g L}^{-1}$) and WW1 ($200 \pm 1 \mu\text{g L}^{-1}$), both from Spectrapure Standards (Oslo, Norway).

In exp #2, Cr concentrations were measured by ICP-MS (iCAPQ, Thermo-Scientific) at $t = 0$ h and $t = 24$ h. Measurements were performed at m/z 52 for solutions amended with Cr salts having NIA and at m/z 50 and 53 for the $^{50}\text{Cr(III)} + ^{53}\text{Cr(VI)}$ mixture. All analyses were performed using Kinetic Energy Discrimination (KED) mode. Because the ICP-MS calibration was performed using solutions with NIA, concentration results for ^{50}Cr and ^{53}Cr were corrected to account for the use of IES. A correction for signal drift in the internal standard was also applied using the following formulas:

$$^{50}\text{Cr}_{\text{corrected}} = (^{50}\text{Cr}_{\text{ICP-MSsoftware}} \cdot 4.345) \cdot (100/\text{baseline drift}) \quad (\text{Eq. 1})$$

$$^{53}\text{Cr}_{\text{corrected}} = (^{53}\text{Cr}_{\text{ICP-MSsoftware}} \cdot 9.500) \cdot (100/\text{baseline drift}) \quad (\text{Eq. 2})$$

$$^{52}\text{Cr}_{\text{corrected}} = (^{52}\text{Cr}_{\text{ICP-MSsoftware}}/\text{baseline drift}) \quad (\text{Eq. 3})$$

Where:

$^{xx}\text{Cr}_{\text{corrected}}$ are the Cr concentrations corrected for isotopic abundances and for the ICP-MS baseline drift (see below);

^{xx}Cr_{ICP-MSsoftware} are the Cr concentrations calculated by the ICP-MS software based on normal isotopic abundances; 'baseline drift' is the ratio between raw counts of the internal standard (⁴⁵Sc) for a given sample divided by the average raw counts for the calibration standards (n = 7) at the beginning of the ICP-MS run. All Cr measurements in exposure solutions were performed on aliquots sampled from aquaria containing living organisms.

Chromium levels in gills and digestive glands of *C. fluminea* were assayed by ICP-MS at m/z 50, 52 and 53 using KED mode after mineralization with 0.5 mL of concentrated nitric acid (65% v/v, Suprapur, VWR) and 0.1 mL of H₂O₂ (30% v/v, Suprapur, VWR). Mineralization was carried out in acid-washed polystyrene tubes placed in a stove at 65 °C overnight. Raw analytical results were corrected as necessary using equations 1 to 3. Gills and digestive glands were recovered by immediately dissecting exposed organisms at the end of the exposure period. Tissues were frozen at -20 °C and then freeze-dried over 24 h (CRIOS-80, Cryotech). The accuracy of the mineralization procedure was checked using the reference material TORT-2 (lobster hepatopancreas) from the National Research Council of Canada (NRC-CNRC). The certified value for total Cr was 0.77 ± 0.15 mg kg⁻¹ (uncertainty expressed as 95% confidence limits). The ICP-MS detection and quantification limits were calculated as three and ten times the standard deviation of replicate analytical blanks (n = 14).

2.4. Biomarker expression

After each exposure period, gills and digestive glands were pooled from two individuals of the same batch to obtain sufficient material for biomarker measurement and stored at -80 °C pending analysis. A total of four samples per exposure conditions was therefore available for biomarker analysis. Immediately before analysis, samples were thawed on ice and their wet weight

determined. Tissues were then transferred to microtubes (2 mL) and manually crushed in 50mM phosphate buffer (98% v/v, pH = 7.6) supplemented with 1% v/v of phenylmethylsulfonyl fluoride (PMSF) and 1% v/v of a 1 mM mixture of L-serine and borate to inhibit proteases (Garaud et al., 2015; Sroda and Cossu-Leguille, 2011). The quantity of added buffer was adjusted to the mass of tissue based on a ratio of 4 mL of buffer to 500 mg of tissue (wet weight). Samples were then centrifuged for 5 min (250 g) at 4 °C. Part of the resulting tissue homogenate was used as such for biomarker analysis. A second aliquot of homogenate was again centrifuged for 20 min (1000 g) at 4 °C. The resulting supernatant was subject to a final centrifugation (50 min, 20000 g, 4 °C) to recover the cytosolic fraction for the measurements of enzymatic activities.

Tissue homogenates were used to assay markers of cellular damage (LOOH, *Lipid Hydroperoxide*; ACP, *Acid phosphatase*), the status of energy reserves (Prot1, *proteins*; TG, *triglycerides*), Cholesterol (Chol.), and the activity of the mitochondrial Electron Transport Systems (ETS). Other markers of oxidative stress (TAC, *Total antioxidant capacity*; GPx-tot, *Glutathione peroxidase*; GST, *Glutathione S-transferase*; CAT, *Catalase*), cellular damage (CSP-3, *Caspase-3*) and energy reserves (Prot2, *proteins*; LDH, *Lactate dehydrogenase*) were measured in the cytosolic fraction. All biomarkers were analysed using an automated spectrophotometer (Konelab 20 XTi, Thermo Scientific) according to the methods described in Garaud et al. (2015), Bertrand et al. (2016) and references therein.

2.5. RNA extraction and RT-qPCR analysis

Gills and digestive glands were excised from 3 individuals per condition after 96 h exposure (exp #1). Non-digestive tissues were carefully removed from digestive glands. Samples were then transferred separately into 1 mL of 4M guanidine thiocyanate and stored at -80° C. Total RNAs were extracted using a RNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's

instructions. Genomic DNA was digested after extraction with DNase I (Qiagen) and a new total RNA purification was performed with RNeasy mini kit. Total RNA purity and quantity were assessed by optical density measurements on a Nanodrop spectrophotometer (A_{260} nm and absorbance ratio 260/280 and 260/230). RNA integrity was evaluated by capillary electrophoresis using a Bioanalyzer 2100 (Agilent, CA, USA).

RT-qPCR was performed on 8 detoxification genes (GST-pi, CAT, Mn SOD, CuZn SOD, TR, MT, HSP70 and Se-GPX) and PS3 as reference gene using the methodologies described in Koehle-Divo et al. (2019) and the sequences reported in Table S2. cDNA was reverse-transcribed from total RNA (900 ng) using the Superscript IV Reverse Transcriptase with oligo(dT)₁₈ according to the manufacturer recommendations (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed on a StepOnePlus PCR system using the Fast SYBR® green master mix (Applied Biosystem®, CA, USA). Amplifications were performed with 50 ng of template, or water (no template control), or 50 ng total RNA (no RT control) using 200 to 300 nM of primers in a final volume of 20 μ L (Koehle-Divo et al., 2019). The cycling conditions were 20 s at 95°C, followed by 40 cycles of 10 s at 95°C (except for CuZn SOD, HSP70, PS3 and GST-pi: 3 s at 95°C) and 30 s at 60° C. A melting curve analysis (65° C–95° C) was performed to ensure the absence of artefacts and the expression of the genes of interest was normalized to that of PS3 using the $2^{-\Delta\Delta C_t}$ method.

2.6. Statistical methods and data treatment

Correlation circle graphs among biomarkers (exp #1 and 2) and detoxification genes (exp #1) were obtained by Principal Component Analysis (PCA) using the R-package ‘FactoMineR’. Biomarkers response was modelled for both exp #1 and 2. For exp #2, accumulation of ‘total chromium’ was added to the list of modelled variables. The values, levels and coding of the

factors considered in the models for the two experiments are presented in Table S3. The levels mentioned in Table S3 were then arranged according to a $2^2 \times 4$ (exp #1) and a 2^3 (exp #2) randomized factorial design with 4 and 3 replicates per combination of the factors in exp #1 and exp #2, respectively. The expression of the 8 mRNAs was also modelled for exp #1, but with a single exposure duration (96h) and three replicates for each factor combination. Parametric statistical methods and regression analysis were performed for biomarkers, mRNAs and Cr accumulation as described below. The homogeneity of variances, associated with all the factor combinations within the replicate groups, was preliminary verified using the modified Levene's test (i.e., Brown-Forsythe's test for homogeneity of variances) after log transformation of the explained variables.

In exp #1, the biomarkers' responses were modelled separately for gills and digestive glands using the following log-linear regression model:

$$\text{Log}_{10}(\text{biomarker}) = \beta_0 + \beta_1 D + \beta_2 \text{Cr} + \beta_3 \text{Co} + \beta_{12} D * \text{Cr} + \beta_{13} D * \text{Co} + \beta_{23} \text{Cr} * \text{Co} + \beta_{123} D * \text{Cr} * \text{Co} \quad (\text{Eq. 4a})$$

where, (D) is the exposure duration, (Cr) indicates the chromium redox form, (Co) the exposure concentration and the '*' sign indicates the interactions between factors (Table S3).

For modelling mRNA responses, Eq. 4a simplified to Eq. 4b because the model did not include exposure duration:

$$\text{Log}_{10}(\text{mRNA}) = \beta_0 + \beta_1 \text{Cr} + \beta_2 \text{Co} + \beta_{12} \text{Cr} * \text{Co} \quad (\text{Eq. 4b})$$

In exp #2, a log-linear regression model was used to describe the response of each biomarker (Eq. 5a) and of total Cr bioaccumulation (Eq. 5b) as a function of the tissue (T) and the presence/absence of trivalent (Cr3) and hexavalent (Cr6) chromium.

$$\text{Log}_{10} (\text{biomarker}) = \beta_0 + \beta_1 T + \beta_2 \text{Cr3} + \beta_3 \text{Cr6} + \beta_{12} T * \text{Cr3} + \beta_{13} T * \text{Cr6} + \beta_{23} \text{Cr3} * \text{Cr6} + \beta_{123} T * \text{Cr3} * \text{Cr6} \quad (\text{Eq.5a})$$

$$\text{Log}_{10} (\text{total Cr}) = \beta_0 + \beta_1 T + \beta_2 \text{Cr3} + \beta_3 \text{Cr6} + \beta_{12} T * \text{Cr3} + \beta_{13} T * \text{Cr6} + \beta_{23} \text{Cr3} * \text{Cr6} + \beta_{123} T * \text{Cr3} * \text{Cr6} \quad (\text{Eq.5b})$$

The testing of the regression coefficients (*i.e.*, the β s') and the subsequent construction of reduced regression models was performed using a stepwise backward procedure with a significance threshold set at $\alpha = 0.01$ for the statistical tests. All statistical analyses were performed using R software (version 3.0.0).

The Cr bioconcentration factors (BCF, in L g^{-1}) were calculated as:

$$BCF = \frac{C_{biota}}{C_{water}} \quad (\text{Eq. 6})$$

where C_{biota} and C_{water} are the concentrations measured in biological material (in $\mu\text{g g}^{-1}$) and in water (in $\mu\text{g L}^{-1}$), respectively.

Chromium speciation calculations were performed using VisualMinteq version 3.1. No changes were made to the default database and the precipitation of amorphous $\text{Cr}(\text{OH})_3$ was allowed as possible solid species. The average composition of Cristaline® mineral water (Table

S1) was used to describe the characteristics of the aqueous matrix and the Debye-Hückel equation was used for activity correction.

3. Results and discussion

3.1. Chromium concentrations and behaviour in test solutions

Figure of merits for FAAS and GFAAS were the same as previously reported (Aharchaou et al., 2018) and analytical blank values were below detection limits. Measured Cr concentration in certified waters ($n = 1$) were $1.96 \mu\text{g L}^{-1}$ for SPS-SW1 (98 % accuracy) and $202 \mu\text{g L}^{-1}$ for WW1 (101 % accuracy). Detection and quantification limits for analyses by ICP-MS were: $^{50}\text{Cr} = 0.074$ and $0.247 \mu\text{g L}^{-1}$, $^{52}\text{Cr} = 0.037$ and $0.124 \mu\text{g L}^{-1}$, $^{53}\text{Cr} = 0.034$ and $0.115 \mu\text{g L}^{-1}$. Analytical blank values were below detection limits for ^{50}Cr and for ^{53}Cr at $t = 0$; between detection and quantification limits for ^{53}Cr at $t = 24$ and measurable for ^{52}Cr . Results for experiments carried out using Cr salts with NIA (for which ^{52}Cr was used) were therefore corrected for the corresponding average analytical blanks. A $200 \mu\text{g L}^{-1}$ standard solution was repeatedly analysed ($n = 5$) during the ICP-MS run with average values of $238 \pm 6 \mu\text{g L}^{-1}$ at $m/z = 52$, $197 \pm 4 \mu\text{g L}^{-1}$ at $m/z = 50$, and $196 \pm 6 \mu\text{g L}^{-1}$ at $m/z = 53$.

Total and filterable Cr concentrations at $t = 0$ were slightly lower than expected for solutions amended with 50 or $500 \mu\text{g L}^{-1}$ in exp #1 and within 15% of the expected values in the other cases (Table S4). Over the 24 h water renewal periods, total and filterable Cr levels varied by less than 15–20 % in the case of Cr(VI), but decreased by 70–95 % in the case of Cr(III) (Figure 1, Table S4). Measurements taken at $t = 0.5, 2, 4$ and 6 h during exp #1 suggest that the concentration decrease began early after Cr(III) addition into the exposure medium (Table S4). The concentrations of the two Cr redox form showed a similar behaviour when added alone or in

combination to the exposure medium (Figure 1; Table S4). A dilution error during the use of IES (see section 2.2) resulted in an initial total filterable Cr(III) concentration of $104 \pm 1.9 \mu\text{g L}^{-1}$ instead of the targeted $50 \mu\text{g L}^{-1}$ value (Figure 1B). Once analytically identified, the higher initial Cr(III) concentration did not prevent the study of the interactions between Cr(III) and Cr(VI) on biomarker expression nor of the simultaneous bioaccumulation of Cr(III) and Cr(VI) by *C. fluminea*.

Figure 1. Panel A: temporal evolution of total (NF) and filterable (F, $< 0.45 \mu\text{m}$) Cr concentrations in solutions amended with $50 \mu\text{g L}^{-1}$ of either Cr(III) or Cr(VI) with natural isotopic abundances (arithmetic means ± 1 standard deviation, $n=3$ for each point). Symbols for total and filterable values have been slightly offset for representation purposes, but samples for measurements were collected at the same time. Panel B: filterable Cr concentrations measured in solutions amended with a mixture of $^{50}\text{Cr(III)}$ ($100 \mu\text{g L}^{-1}$) and $^{53}\text{Cr(VI)}$ ($50 \mu\text{g L}^{-1}$) using isotopically enriched solutions. See Table S4 for tabulated data.

The decrease in total and filterable chromium concentrations in all solutions amended Cr(III) agrees with the known tendency of Cr(III) to form insoluble Cr(OH)_3 species at circumneutral pH (Rai et al., 1989). Speciation calculation predicted that approx. 81 % and 98 % of the added

Cr(III) would precipitate for initial concentrations of 50 and 500 $\mu\text{g L}^{-1}$. According to VisualMinteq calculations, the theoretical maximum soluble Cr(III) concentration in Cristaline® water is about 10 $\mu\text{g L}^{-1}$ at a pH of 7.4 (measured at the source); in reasonable agreement with experimentally measured Cr concentrations at 24 hours (Fig. 1 and Table S4). On the other hand, the model does not predict the formation of any precipitate for added Cr(III) concentrations of 5 $\mu\text{g L}^{-1}$, in disagreement with the experimental results (Table S4).

Because VisualMinteq is a thermodynamic model, the agreement between theoretical solubility and experimentally measured concentrations at $t = 24$ h suggests that the exposure system reached quasi equilibrium within 24 hours. The decrease in filterable Cr concentrations agrees with the hypothesis of a rapid formation of Cr(III) bearing particles in the test medium (Aharchaou et al., 2018). On the other hand, the decrease in total Cr concentrations suggests that other phenomena also occurred during the experiments (*e.g.*, adsorption onto the vessels' walls and on the organisms' shells). These phenomena may also explain the observed concentration decrease in solutions initially amended with 5 $\mu\text{g L}^{-1}$ of Cr(III); *i.e.* a concentration below the theoretical solubility limit.

3.2. Chromium bioaccumulation and bioavailability

Chromium levels in control organisms were above the analytical quantification limits (see section 3.1) only for ^{52}Cr : $0.74 \pm 0.17 \mu\text{g g}^{-1}$ for gills (G) and $0.32 \pm 0.09 \mu\text{g g}^{-1}$ for digestive glands (DG). Results for Cr accumulation were therefore corrected for the corresponding blank values in specimen exposed to solutions prepared using Cr salts with NIA (for which measurements were performed at m/z 52). No blank corrections were necessary for specimen exposed to $^{50}\text{Cr(III)}$ and $^{53}\text{Cr(VI)}$ using IES. Duplicate analysis of the reference material TORT2

gave blank corrected Cr concentrations (measured at $m/z = 52$) of 0.643 and $0.740 \mu\text{g g}^{-1}$ corresponding to percentage recoveries of 83 and 96 %, respectively.

For specimen exposed to individual Cr redox forms, Cr(III) accumulation was higher in DG than in G (10.2 ± 5.5 vs. $2.98 \pm 1.05 \mu\text{g g}^{-1}$), while the opposite situation was observed for Cr(VI) (2.64 ± 1.11 vs. $12.9 \pm 0.19 \mu\text{g g}^{-1}$) (Fig. 2). In organisms exposed to the Cr(III) and Cr(VI) mixture, Cr accumulation was higher in G than in DG for both redox forms (Mann-Whitney-Wilcoxon test; $P=0.029$).

Figure 2. Arithmetic means of Cr concentrations accumulated in gills (hatched bars) and digestive glands (full bars) of individuals of *Corbicula fluminea* exposed for 96 hours to solutions amended with either Cr(III) or Cr(VI) salts having natural isotopic abundances or to a solution simultaneously amended with a mixture of $^{50}\text{Cr(III)}$ and $^{53}\text{Cr(VI)}$ using isotopically enriched solutions (see Table S4, exp #2, for actual exposure concentrations in each scenario). Errors are expressed as ± 1 standard deviation. Analytical results for individual samples are given in Table S5. NIA, solutions with Natural Isotopic Abundances; IES, Isotopically Enriched Solutions.

The fitted bioaccumulation model (Eq. 5b) obtained from experimental data was:

$$\text{Log}_{10}(\text{total Cr}) = 0.405 - 0.164 T + 0.249 \text{Cr3} + 0.286 \text{Cr6} + 0.092 T*\text{Cr3} - 0.166 T*\text{Cr6} - 0.271 \text{Cr3}*\text{Cr6} \quad (\text{Eq. 7})$$

with a high explanatory power and predictive capacity (F test, $P < 10^{-4}$; $r^2 = 0.954$; coefficient of variation of the residuals = 34.11%). Except for the three-way interaction $T*\text{Cr3}*\text{Cr6}$ ($P=0.03$), all factors had a statistically significant effect ($P<0.01$) on the bioaccumulated total Cr concentration and could be ranked as follows: $\text{Cr6} \geq \text{Cr3}*\text{Cr6} \geq \text{Cr3} > T*\text{Cr6} \geq T > T*\text{Cr3}$. The results of the fitted model agreed well with the experimentally measured total Cr concentrations (Table 1).

The fitted bioaccumulation model (Eq. 7) suggests that both direct and interaction effects control Cr(III) and Cr(VI) bioaccumulation by *C. fluminea*. The interaction effects between the Cr redox forms and the tissues (e.g., $T*\text{Cr3}$ and $T*\text{Cr6}$) agree with the experimental observation that exposure to Cr(III) or Cr(VI) led to a preferential accumulation of Cr in DG or G, respectively. Equation 7 also suggests the presence of an antagonistic effect between Cr(III) and Cr(VI) (e.g., $\text{Cr3}*\text{Cr6}$) with a magnitude (i.e., the value of the coefficient in Eq. 7) being comparable to the direct effects of both redox forms. This antagonistic effect is confirmed by the experimental results (Table 1). Specifically, the accumulation of Cr(VI) decreased by about 50% in both G and DG of organisms exposed to the Cr(III)+Cr(VI) mixture compared with the Cr(VI)-only exposure. The presence of Cr(VI) did not affect Cr(III) levels in G, but lowered Cr(III) accumulation in DG by about 10-fold. This antagonistic effect is unlikely to arise from differences in the uptake of the three Cr isotopes considered in the present study. Indeed, in

specimen exposed to Cr(VI) or Cr(III) solutions prepared using salts with NIA, the coefficient of variation of the measured Cr concentrations obtained at m/z 50, 52 and 53 was between 8 and 12%; *i.e.*, not large enough to account for the observed decreases in Cr(III) and Cr(VI) bioaccumulation during mixture exposure.

Although the cellular uptake mechanisms for Cr(VI) and Cr(III) are known to differ (Beyersmann and Hartwig, 2008; Viti et al., 2014), further research is required to understand the mechanisms behind the observed antagonist effect. Future studies should also clarify if the accumulation of Cr(III) solely reflects absorption of soluble elemental species inside the tissues or if adsorption/absorption of Cr-bearing nanoparticles (see section 3.1) also contributes to the Cr body burdens of *C. fluminea*. The role of Cr-bearing nanoparticles in Cr(III) bioaccumulation may have a real environmental relevance because polymeric Cr(III) species can occur in environmental samples (Hu et al., 2016). Previous studies on Cr bioaccumulation by other bivalves confirm the bioavailability of Cr(VI) and Cr(III) from dissolved or particulate pathways (Parlak et al., 1999; Roditi and Fisher, 1999; Wang et al., 1997). Extending the isotopic approach to simultaneous exposure to dissolved Cr(VI) and particulate-bound Cr(III) would allow to better understand Cr bioaccumulation in real field conditions where simultaneous exposure to Cr(III) and Cr(VI) can occur.

Table 1. Total chromium concentrations (95% confidence intervals are given in brackets) accumulated in gills and digestive glands for each combination of factors in experiment 2 as estimated by the fitted reduced model described in Eq. 7. The corresponding arithmetic means of measured concentrations (in italics) are reported for comparison purposes (see Table S5). Nominal exposure concentrations were 0 or 50 $\mu\text{g L}^{-1}$ except for ‘\$’ that was 100 $\mu\text{g L}^{-1}$ (see

section 2.2). Concentrations in organisms exposed to either Cr(III) or Cr(VI) have been corrected for the corresponding blank values of control organisms, while no blank corrections were necessary for organisms exposed to the Cr(III)+Cr(VI) mixture (see section 3.2).

Cr(III)	Cr(VI)		Total accumulated Cr concentration ($\mu\text{g g}^{-1}$)			
			Gills		Digestive glands	
			Mean	95% CI	Mean	95% CI
no	no	Estimated	0.488	[0.335; 0.710]	0.323	[0.222; 0.471]
		<i>Measured</i>	<i>0.737</i>	---	<i>0.319</i>	---
no	yes	Estimated	13.6	[9.36; 19.8]	1.96	[1.35; 2.85]
		<i>Measured</i>	<i>12.9</i>	---	<i>2.64</i>	---
yes	no	Estimated	3.51 [‡]	[2.41; 5.1]	5.42	[3.72; 7.88]
		<i>Measured</i>	<i>2.98</i>	---	<i>10.2</i>	---
yes ^s	yes	Estimated	8.06	[5.54; 11.7]	2.70	[1.85; 3.93]
		<i>Measured</i>	<i>8.80^A</i>	---	<i>2.02^A</i>	---
			<i>2.49^B</i>	---	<i>0.737^B</i>	---
			<i>6.31^C</i>	---	<i>1.28^C</i>	---

A, total Cr = ⁵⁰Cr(III) + ⁵³Cr(VI); B, ⁵⁰Cr(III); C, ⁵³Cr(VI)

[‡] Example of calculation using Eq. 7. Remembering that the actual numerical values of the factors Cr3, Cr6 and T in Eq. 7 correspond to the codes in Table S3, total Cr accumulation in gills (code = -1) for organisms exposed to 50 $\mu\text{g L}^{-1}$ of Cr(III) (code = +1) and 0 $\mu\text{g L}^{-1}$ of Cr(VI) (code = -1) leads to the following development for Eq. 7: $\text{Log}_{10}(\text{total Cr}) = 0.405 - [0.164 \times (-1)] + [0.249 \times (+1)] + [0.286 \times (-1)] + [0.092 \times (-1) \times (+1)] - [0.166 \times (-1) \times (-1)] - [0.271 \times (+1) \times (-1)] = 0.545$; or 3.51 $\mu\text{g g}^{-1}$ on a linear scale.

Organisms exposed to Cr(III) showed a higher BCF in DG than in G (0.5 vs. 0.15 L g⁻¹), while the opposite was true for Cr(VI) (0.05 vs. 0.22 L g⁻¹). In exposures to the Cr(III) + Cr(VI) mixture, both redox forms showed higher BCFs in G than in DG: 0.08 vs. 0.02 for Cr(III) and 0.14 vs. 0.03 for Cr(VI) (all values in L g⁻¹). Calculations of BCF for Cr(III) were done using the geometric means of the measured filterable concentrations at t = 0 and t = 24 h to account for the temporal decrease in Cr concentration (Fig. 1, Table S4). Existing studies on Cr accumulation in bivalves are biased toward Cr(VI) or do not specify Cr speciation in the exposure matrices (Table S6). Potet et al. (2016) exposed two species of dreissenids to Cr(VI) using experimental conditions similar to those adopted in the present study. Despite the differences in the measured Cr burdens, the BCF factors for the two dreissenids (Table S6) are similar to those observed in the present study for *C. fluminea* exposed to Cr(VI). de Oliveira and dos Reis Martinez (2014) exposed specimen of *C. fluminea* along a contamination gradient in a stream impacted by landfill leachate and observed BCF much higher than those reported in the present study (Table S6). We surmise that, during such in situ exposures, the bulk of accumulated Cr originated from particle ingestion rather than uptake of aqueous Cr species. Uptake of particle-bound Cr(III) has been confirmed using radiolabelled material in laboratory studies (Roditi and Fisher, 1999; Wang et al., 1997). Comparison with other published results is less straightforward because of interspecies differences and variability of exposure conditions (Table S6). Nonetheless, our results clearly highlight the need for more research on the bioavailability of Cr(III) or Cr(III) + Cr(VI) mixtures.

3.3. Biomarker responses

Biomarkers expression could be properly measured except for TAC in gills (both experiments) and CAT in gills and digestive glands (exp #2 only). The expression of energy related biomarkers (Prot 1, Prot 2, TG, LDH), Chol and some markers of oxidative stress (TAC, GST and CAT) was

higher in digestive glands (DG) than in gills (G) for both experiments and regardless of exposure conditions (Tables S7 and S8). Among the markers of cellular damage, ACP activities were similar in the two tissues, while CSP-3 levels were higher in G than in DG for both experiences. Lipid hydroperoxide (LOOH) behaved differently in exp #1 (DG > G) and exp #2 (G > DG); most likely because of the changes in LOOH levels among the specimen of *C. fluminea* not expose to Cr between the two experiments (Tables S7 and S8). In some cases, the experimental values were too low for quantification (blank cells in Tables S7 and S8) and the corresponding results were excluded from further statistical analysis.

For exp #1, the first two PCA factors explained 58.2% and 66.3% of the inertia for G and DG, respectively. Apart from Chol, Prot1, Prot2, TG and CAT, LDH, the associations between the biomarkers differed between tissues (Fig. 3). Note however that CAT and LDH were negatively correlated in G and positively correlated in DG. ACP and CSP3 were associated with Chol, Prot1, Prot2, TG in G and with CAT and LDH in DG. LOOH was linked to either GST (G) or ETS (DG) and seemed to respond differently to Cr exposure compared with other biomarkers. In DG, the first PCA axis opposed some energy-related biomarkers (Prot, TG) and Chol to those indicative of oxidative or cellular damage (Fig. 3B).

Regression analysis did not yield statistically significant models for Chol, LDH, TG and, on a tissue-specific basis Prot 1, Prot 2, GST and LOOH (Table S9). These biomarkers appear unsuitable to detect adverse effects of Cr in G and/or DG of *C. fluminea*. Regression analysis was not performed for CAT and TAC because the missing values prevented comparisons between tissues and experiments. Statistically significant direct effects of Cr redox form and/or Cr concentration were observable for ACP and CSP3 in gills, while exposure duration had a direct effect on ACP in gills and ETS in both gills and digestive glands (Table S7). The responses of other biomarkers in exp #1 were mostly driven by interaction effects among exposure duration,

Cr redox state and Cr exposure concentration (Table S9). In particular, interaction effects involving Cr(VI) or Cr(III) concentration were identified for Prot1, LOOH, and CSP3 in DG and Prot2, ACP, CSP3 and ETS in G (Table S9). In the other cases, interaction effects involved only exposure duration and/or Cr oxidation state.

Figure 3. Correlation circle graphs of the PCA (A: gills; B: digestive glands) for the biomarker activities measured in specimen of *Corbicula fluminea* exposed to Cr(III) and Cr(VI) in experiment 1.

Interpreting interaction effects is a complex task and is better illustrated graphically for some biomarkers with the highest r^2 ; e.g. ETS and CSP3 in G and ETS and LOOH in DG (Table S9 and Fig. 4). The activities of ETS in G at 96 h decreased with increasing Cr(III) concentrations and increased with increasing Cr(VI) concentrations, but showed no clear trends at 24 h. The response of CSP3 to Cr(III) showed opposite trends at 24 h and 96 h, while CSP3 levels in organisms exposed to Cr(VI) increased with concentration regardless of exposure duration. In DG, ETS levels peaked in specimen exposed to $50\mu\text{g L}^{-1}$ Cr(III) for 96 h, but no clear trends

emerged for the other exposure conditions. Similarly, LOOH expression increased monotonically with Cr(III) concentration for 96 h exposures, but otherwise had variable patterns.

Figure 4. Interaction plots for the responses of ETS and CSP3 in gills (A; top graphs) and of ETS and LOOH in digestive glands (B; bottom graphs) for different combinations of the Cr redox forms and the exposure time, as a function of Cr concentration in experiment 1.

Wang et al. (2012) observed that exposure to Cr(VI) increased lipid peroxidation and decreased catalase activity in *C. fluminea*. Effects of Cr(VI) exposure on various biomarkers have been previously reported in *Mytilus galloprovincialis* and *Dreissena* spp. (Barmo et al., 2011; Ciacci et al., 2011; Ciacci et al., 2012; Potet et al., 2016). The present study suggests that Cr(III) can also affect biomarker expression in bivalves and should be considered as a biologically active chromium form deserving more attention in future studies.

For exp #2, neither Cr(III) nor Cr(VI) showed any direct effect on biomarker responses, but differences in biomarker expression existed between tissues. Expression levels of Chol, TG, Prot1, Prot2 and GST were significantly higher ($P < 10^{-4}$) in DG than in G, while the opposite ($P < 0.01$) was observed for LOOH, CSP3 and ETS (Table S9). The simultaneous exposure to Cr(III) and Cr(VI) did not seem to modify biomarkers responses compared with exposure to individual redox forms of Cr; at least for the selected aqueous exposure levels and the corresponding bioaccumulated concentrations (see section 3.5).

3.4. Gene expression

The expression levels of all mRNAs could be properly quantified except for Se-GPX in G. The first two PCA factors explained 75% and 72% of the inertia for the G and DG, respectively (Fig. 5). The expression patterns of mRNAs differed between the two tissues. For example, the correlations between Mn SOD and CAT as well as TR and CuZn SOD were observed only in G, while Mn SOD and MT were correlated only in DG.

Regression models (Table S10) also pointed to tissue-dependent relationships between mRNA expression and Cr exposure. In the gills, expression of mRNAs for GST-pi, CAT, CuZn SOD, Mn SOD, MT and HSP70 increased with Cr(III) and slightly decreased with Cr(VI) concentrations. In digestive glands, significant regression models were obtained only for TR, MT

and HSP70. The expression of TR and HSP70 increased with Cr concentration for both redox states, although the response was higher in the presence of Cr(VI). At the opposite, MT expression decreased with Cr concentration regardless of the element oxidation state (Table S10).

Figure 5. Correlation circle graphs of the PCA (A: gills; B: digestive glands) for the mRNA expression in specimen of *Corbicula fluminea* exposed to Cr(III) and Cr(VI) in experiment 1.

Changes in the expression levels of GST-pi, CAT, MnSOD, CuZnSOD, MT, TR and HSP70 were driven by both Cr redox state and concentration in at least one tissue, while MT expression in digestive glands was driven only by Cr concentration (Table S10). The effects on mRNA expression are mostly marked in the gills compared to the digestive glands (Table S10). Modifications of GST-pi, CAT, MT and HSP70 mRNA levels have been reported in the gills and digestive glands of *Mytilus galloprovincialis* exposed to Cr(VI) (Ciacci et al., 2012; Franzellitti et al., 2012), while we are unaware of previous report for Cr(III) effects on mRNA expression in *C. fluminea* or other bivalves. Ciacci et al. (2012) also observed that the effects of Cr(VI) on mRNA

GST-pi, CAT, MT isoforms and HSP70 differed between male (decreased expression) and female (increased expression); a distinction that was not taken into account in the present study.

5. Conclusions

Both Cr(III) and Cr(VI) can accumulate in gills and digestive glands of the freshwater mussel *Corbicula fluminea* and affect biomarkers' and mRNA expression. Future research on Cr in bivalves and, likely, other aquatic organisms should therefore consider both redox forms and not focus exclusively on Cr(VI). Exposure to Cr(III)+Cr(VI) mixtures does not markedly modify the accumulation of Cr(III) and Cr(VI) in gills compared with exposures to individual redox forms. On the other hand, the presence of Cr(VI) seems to decrease Cr(III) accumulation in digestive glands. Typical laboratory-based scenarios may therefore miss possible interactions between Cr(VI) and Cr(III) when exposing organisms to individual Cr redox forms. This caveat applies to the study of Cr uptake, bioaccumulation and ecotoxicity in both laboratory and real field conditions. Studies under more diversified exposure scenarios, including exposure via dietary pathways, are required to confirm the general validity of this hypothesis.

The regression approach suggests that biomarkers for the activity of mitochondrial electron transport systems (ETS) and cellular damage (ACP and CSP-3) are the most suitable to study the effect of Cr on *C. fluminea*. However, the biomarker response varies depending on the selected tissue and must also consider the complex interactions between Cr oxidation state, Cr concentration and exposure duration. In the case of antioxidant genes, regression analysis suggests that CAT and MnSOD mRNA levels are the most relevant to study in the gills. For digestive glands, regression for mRNA levels of TR and HSP70 were the most significant. In all

cases, regression analysis suggest that mRNA levels respond to both Cr(III) and Cr(VI), further supporting the recommendation for more comparative studies on both Cr oxidation states.

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CRediT author statement

IA: Conceptualization, Investigation, Validation, Writing (Original Draft)

AM: Formal analysis, Visualization, Writing (Original Draft)

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DP: Investigation

HP: Investigation

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Supporting information for

Effects and bioaccumulation of Cr(III), Cr(VI) and their mixture in the freshwater mussel

Corbicula fluminea

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833 Table S8. Arithmetic means $\pm$ 1 standard deviation (n = 4; n = 3 for \$) for the values of cellular
834 biomarkers measured in gills and digestive glands during exp # 2.

835 Table S9. Regression analysis of the biomarkers' expression

836 Table S10. Regression analysis of the mRNA expression

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858 Table S1: Composition of Cristaline® mineral water (spring Aurèle) used as a test medium for
859 experiments on biomarker activities in and Cr accumulation by specimen of *Corbicula fluminea*.
860 All values are in mg L⁻¹.

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Element	Concentration
Ca	106
Mg	4.2
Na	3.5
K	1.5
HCO ₃	272
Cl	3.8
SO ₄	50
NO ₃	1
Si	8.3
F ⁻	0.9
pH	7.2

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879 Table S2. Primer sequences used in RT-qPCR analysis.

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Gene Name	Accession Number (GenBank)	Forward (5'-3')	Reverse (5'-3')	Amplicon length (bp)	Concentration used for qPCR (nM)	GO biological process	Ref.
Catalase	EF446609	CCTTCAGTGCCTAGTCTGG	CCTGTTCTCTGACCGTGG	196	200	Response to oxidative stress	1
Cu-Zn Superoxide dismutase	KF218347.1	GAATGTTGTGGCTGATGAGG	GGATCGTTCTCTGTCAATG	76	250	Response to oxidative stress	2
Se-GPx	EF547366.1	TATGCCCTGTTCCCGTGTC	CTGCCATCTGGACCAATCAG	148	200	Response to oxidative stress	3
GST pi	AN AY885667.1	CTGGGCGACCGAATACAAATC	GATGGCATTGGACTGGGCTA	90	250	Response to reactive oxygen species	3
HSP70	KJ461738.1	AAGAAGGTTGGTGGTGAACG	TCCCAAATGTGTGTCTCCTG	121	250	Cellular response to stress, heat, protein refolding	4
Metallothionein (isoform 1)	EF185126	CGGCTATCTCCCGCGA	AGCTTTTACCAGAACCAACAGT	210	200	Cellular metal ion homeostasis, cellular response to oxidative stress	1
Mn Superoxide dismutase	EF446611	CTGTAGGTAGTAAGCGTGCTCC	GGATCATTTGACGAGATGAAGGC	200	200	Response to oxidative stress	1
Ribosomal protein S3	AJ517687	CAGTGTGAGTCCCTGAGATACAAG	AAACGGTTGATAGGACGCAAG	154	250	Translation (Used as housekeeping gene)	5
Thioredoxin reductase	KF275126	AATGCGGTGGCTTCCTACA	TCTGCTGGTCAAATCCTCGC	89	200	Cell redox homeostasis	3

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882 ¹Arini A, Daffe G, Gonzalez P, Feurtet-Mazel A, Baudrimont M. 2014. Detoxification and recovery capacities of *Corbicula fluminea*
883 after an industrial metal contamination (Cd and Zn): a one-year depuration experiment. *Environ Pollut.* 192, 74-82.

884 ²Koehle-Divo V, Pain-Devin S, Bertrand C, Devin S, Mouneyrac C, Giamberini L, Sohm B. 2019. *Corbicula fluminea* gene expression
885 modulated by CeO₂ nanomaterials and salinity. *ESPR.* 26(15), 15174-15186.

886 ³Chen H, Zha J, Yuan L, Wang Z. 2015. Effects of fluoxetine on behavior, antioxidant enzyme systems, and multixenobiotic resistance
887 in the Asian clam *Corbicula fluminea*. *Chemosphere.* 119, 856-62.

888 ⁴Unpublished probes

889 ⁵Navarro A, Faria M, Barata C, Piña B. 2011. Transcriptional response of stress genes to metal exposure in zebra mussel larvae and
890 adults. *Environ Pollut.* 159, 100-7.

Table S3. Factors, levels and coding values used for regression modelling in the two experiments. Tissue was not considered as a factor in experiment 1 because data for gills and digestive glands were treated separately, while only one exposure duration (96 h) was used in experiment 2 (see text, section 2.2, for details). In the column ‘Factor’, Cr3 and Cr6 are the chosen variable names for the corresponding Cr redox species.

	Factor	Level	Coding
Experiment 1	Exposure duration (D)	24h	-1
		96h	1
	Chromium redox form (Cr)	Cr3	-1
		Cr6	1
Experiment 2	Exposure concentration (Co)	0 $\mu\text{g L}^{-1}$	0
		5 $\mu\text{g L}^{-1}$	5
		50 $\mu\text{g L}^{-1}$	50
		500 $\mu\text{g L}^{-1}$	500
Experiment 2	Tissue (T)	Gills	-1
		Digestive glands	1
	Cr3	0 $\mu\text{g L}^{-1}$	-1
		50 $\mu\text{g L}^{-1}$	1
Experiment 2	Cr6	0 $\mu\text{g L}^{-1}$	-1
		50 $\mu\text{g L}^{-1}$	1

910 Table S4. Total and total filterable (< 0.45 µm) Cr concentrations in aliquots of Cristaline® water amended with different levels of either Cr(III)
 911 or Cr(VI) at different incubation times (in hours).

912
 913 **Experiment 1.** All values are in µg L⁻¹. Concentrations at t=0, 2, 6 and 24 hours are arithmetic means ± one standard deviations of triplicate
 914 measurements (duplicate measurements for t = 2). Concentrations at t = 0.5, 1 and 4 hours were measured only in one replicate. Bdl, below
 915 detection limit.
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Expected concentrations		Measured concentrations						
		t = 0	t = 0.5	t = 1	t = 2	t = 4	t = 6	t = 24
Cr(III) control unfiltered	0	0.19 ± 0.12	0.07	bdl	bdl	bdl	0.19 ± 0.27	1.0 ± 1.4
Cr(III) control <0.45 µm	0	0.07 ± 0.11	bdl	bdl	0.35 ± 0.49	bdl	0.17 ± 0.28	0.97 ± 1.27
Cr(VI) control unfiltered	0	0.07 ± 0.06	bdl	bdl	bdl	bdl	0.08 ± 0.10	0.42 ± 0.40
Cr(VI) control <0.45 µm	0	0.10 ± 0.17	bdl	bdl	bdl	bdl	0.07 ± 0.06	0.24 ± 0.33
Cr(III) unfiltered	5	4.59 ± 0.27	3.85	3.03	2.26 ± 0.23	1.41	1.64 ± 0.46	1.18 ± 0.29
Cr(III) filtered 0.45 µm	5	4.44 ± 0.41	3.73	4.09	2.33 ± 0.18	1.37	1.54 ± 0.45	0.68 ± 0.46
Cr(VI) unfiltered	5	4.32 ± 0.36	3.99	3.93	4.22 ± 0.67	3.97	4.32 ± 0.48	3.64 ± 0.44
Cr(VI) filtered 0.45 µm	5	4.93 ± 0.35	4.24	4.32	4.48 ± 0.44	4.24	4.50 ± 0.42	4.13 ± 0.40
Cr(III) unfiltered	50	31.3 ± 4.04	26	24	22.0 ± 2.8	15	17.3 ± 2.9	8.33 ± 2.08
Cr(III) filtered 0.45 µm	50	30.7 ± 1.53	29	24	22.0 ± 1.4	13	14.7 ± 2.5	6.00 ± 1.00
Cr(VI) unfiltered	50	36.0 ± 5.00	31	32	33.5 ± 4.9	30	32.7 ± 3.2	29.3 ± 3.1
Cr(VI) filtered 0.45 µm	50	34.3 ± 2.52	31	33	31.5 ± 4.9	31	33.7 ± 3.2	30.3 ± 2.5
Cr(III) unfiltered	500	434 ± 86.0	309	281	228 ± 2	136	124 ± 24	246 ± 210
Cr(III) filtered 0.45 µm	500	362 ± 25.4	304	274	148 ± 16	83	55.7 ± 9.7	18 ± 5.6
Cr(VI) unfiltered	500	363 ± 26.8	354	351	363 ± 30	327	361 ± 26	312 ± 32
Cr(VI) filtered 0.45 µm	500	378 ± 20.8	361	351	364 ± 28	336	360 ± 28	318 ± 28

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922 **Experiment 2.** NIA, solutions with natural Cr isotopic abundances; IES, isotopically enriched solutions. Concentration in control exposures are
923 given for ^{52}Cr only considering that only the concentration of this isotope was above the ICP-MS detection limit (see main text, section 3.1 for
924 details). All values are arithmetic means $\pm$ 1 standard deviations, n = 3, t is in hours.
925

	Expected concentration	Measured concentration	
		t = 0	t = 24
^{52}Cr control unfiltered	0	0.84 ± 1.29	0.91 ± 1.33
^{52}Cr control filtered 0.45 μm	0	0.43 ± 0.43	0.37 ± 0.49
$^{52}\text{Cr(III)}$ unfiltered – NIA	50	56.4 ± 2.98	16.1 ± 3.59
$^{52}\text{Cr(III)}$ filtered 0.45 μm – NIA	50	56.2 ± 0.93	7.35 ± 0.81
$^{52}\text{Cr(VI)}$ unfiltered – NIA	50	56.0 ± 3.89	52.6 ± 1.81
$^{52}\text{Cr(VI)}$ filtered 0.45 μm – NIA	50	58.5 ± 1.76	52.7 ± 2.81
$^{50}\text{Cr(III)}$ unfiltered – IES	100	108 ± 6.2	26.7 ± 4.92
$^{50}\text{Cr(III)}$ filtered 0.45 μm – IES	100	104 ± 1.9	9.89 ± 1.98
$^{53}\text{Cr(VI)}$ unfiltered – IES	50	45.0 ± 1.86	42.0 ± 0.32
$^{53}\text{Cr(VI)}$ filtered 0.45 μm – IES	50	45.0 ± 0.15	42.0 ± 1.13

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935 Table S5. Chromium concentrations accumulated in the gills (G) and digestive glands (DG) of individual specimen of *Corbicula fluminea*
936 exposed for 96 hours to solutions amended with either Cr(III) or Cr(VI) salts having natural isotopic abundances (NIA) or to a solution
937 simultaneously amended with a mixture of ⁵⁰Cr(III) and ⁵³Cr(VI) using isotopically enriched solutions (IES). n.m., isotope not measured.

Cr species/isotopes	Exposure Concentration (µg L ⁻¹)	Tissue	⁵² Cr (µg g ⁻¹)	⁵⁰ Cr(III) (µg g ⁻¹)	⁵³ Cr(VI) (µg g ⁻¹)
Cr(III) – NIA	50	Gills	2.034	3.192	2.611
			4.381	5.069	4.615
			2.332	3.105	2.727
			3.161	3.469	3.222
		DG	4.998	5.097	4.257
			6.191	7.455	6.398
			13.250	13.984	11.140
			16.293	19.656	16.797
Cr(VI) –NIA	50	Gills	13.058	12.445	11.856
			12.783	11.099	11.057
		DG	2.490	2.318	2.234
			3.812	3.533	3.399
			1.613	1.656	1.508
⁵⁰ Cr(III) + ⁵³ Cr(VI) – IES	100 + 50	Gills	n.m.	2.528	8.038
			n.m.	2.923	7.692
			n.m.	1.891	5.582
			n.m.	2.616	3.911
		DG	n.m.	0.969	1.134
			n.m.	0.232	1.437
			n.m.	1.394	2.002
			n.m.	0.354	0.539

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941 Table S6. Comparison of Cr bioaccumulation ($\mu\text{g g}^{-1}$ dry weight) in bivalves under different exposure conditions from published literature
 942 studies. \$, exposure temperature = 12°C ; #, exposure temperature = 17°C ; & = $\mu\text{g g}^{-1}$ wet weight ; BCF = Bioconcentration factor.
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Exposure conditions						Bioaccumulation		
Organism	Acclimation (d)	Cr form	Cr (µg L ⁻¹)	Duration (d)	Test medium	Tissue	Cr (µg g ⁻¹)	BCF (L g ⁻¹)
<i>Mytilus galloprovincialis</i> ¹	3	Cr(VI)	0.001	7	Filtered seawater	DG	1.5	1500
			0.01				2.5	250
			0.05				2.7	54
			0.001			Soft tissue (no DG)	1	1000
			0.01				1.2	120
			0.05				2.2	44
<i>Dreissena polymorpha</i> ²	4	Cr(VI)	5	4 ^{\$}	Cristaline® mineral water	Whole organism (no shell)	0.5 ± 0.2	0.1
			15				1.9 ± 0.7	0.13
			5	4 [#]			0.7 ± 0.1	0.14
			15				1.3 ± 0.7	0.09
			5	4 ^{\$}			1 ± 0.2	0.2
			15				0.9 ± 0.4	0.06
<i>Dreissena r. bugensis</i> ²			5	4 [#]			1.2 ± 0.3	0.24
			15				1.2 ± 0.3	0.08
			<i>Mytilus galloprovincialis</i> ³				7	Cr(VI)
5	2.5 ± 0.4	0.5						
50	15.4 ± 6.1	0.31						

Table S6 (continued)

Organism	Acclimation (d)	Cr form	Cr (μg L ⁻¹)	Duration (d)	Test medium	Tissue	Cr (μg g ⁻¹)	BCF (L g ⁻¹)					
<i>Caelatura companyoti</i> ⁴	15	Unspecified	20000	1	Unspecified	Whole organism (no shell)	3400 ^{&}	3400					
				2			8400	4200					
				5			16800	3360					
				10			28200	2820					
				15			42400	2827					
				20			65000	3250					
<i>Crassostrea virginica</i> ⁵	23	unspecified	88	4	Artificial seawater	G	27.47 ± 18.65	0.31					
			DG			13.89 ± 5.69	0.16						
			G			19.41 ± 2.87	0.13						
			DG			28.19 ± 12.85	0.2						
<i>Crassostrea virginica</i> ⁶	1 month	Cr(VI)	5	12 weeks	Artificial seawater	Whole organism (no shell)	3.12 ± 0.45	0.62					
			10				5.63 ± 1.15	0.56					
<i>Mytilus edulis</i> ⁶			5				4.83 ± 1.32	0.97					
			10				9.41 ± 3.37	0.941					
			<i>Corbicula fluminea</i> ⁷				0	Cr total	0.70 – 3.58	In situ (river)	G	8.24 – 22.59	2.3 – 21 ^A
									5			17.51 – 31.44	4.9 – 45 ^A
15	29.83 – 34.32	8.3 – 49 ^A											
30	22.75	6.3 – 32 ^A											
0.70 – 3.58	DG	4.05 – 9.05		1.1 – 13 ^A									
		3.28 – 14.81		0.9 – 21 ^A									
		4.80 – 17.31		1.3 – 25 ^A									
		3.51 – 18.22		1.0 – 26 ^A									

945 A, the BCF range covers the ratios (minimum accumulated concentration / maximum exposure concentration) and (maximum accumulated
 946 concentration / minimum exposure concentration).

947

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- 959 6. Zaroogian GE, Johnson M. Chromium uptake and loss in the bivalves *Crassostrea virginica* and *Mytilus edulis*. Marine Ecology Progress
960 Series. 1983;12:175-84.
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962 Landfill Leachate Contamination. Bulletin of Environmental Contamination and Toxicology. 2014;93(2):149-53.
- 963

964 Table S7. Arithmetic means $\pm$ 1 standard deviation (n = 4; n = 3 for \$) for the values of cellular biomarkers measured in gills and digestive
965 glands of the freshwater bivalve *Corbicula fluminea* exposed to 0, 5, 50 and 500 $\mu\text{g L}^{-1}$ of Cr(III) or Cr(VI) during 24 or 96 h (exp #1). w.w., wet
966 weight.
967

Biomarker	Exposure (Type)	Exposure (Duration)	Gills				Digestive glands			
			0 $\mu\text{g L}^{-1}$	5 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$	500 $\mu\text{g L}^{-1}$	0 $\mu\text{g L}^{-1}$	5 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$	500 $\mu\text{g L}^{-1}$
Protein content 1 (homogenate) mg/g w.w.	Cr(III)	24 h	19.1 $\pm$ 3.5	18.7 $\pm$ 3.5	17.3 $\pm$ 4.9	18 $\pm$ 4.6	38.7 $\pm$ 1.5	36.0 $\pm$ 6.4	37.1 $\pm$ 6.3	41.4 $\pm$ 4.8
	Cr(VI)		16.1 $\pm$ 4.8	18.9 $\pm$ 2.8	18.7 $\pm$ 1.3	18.1 $\pm$ 1	35.9 $\pm$ 7.2	44.1 $\pm$ 7	37.9 $\pm$ 4.8	31.0 $\pm$ 5.3
	Cr(III)	96 h	20.4 $\pm$ 1.3	18.1 $\pm$ 1.2	16.9 $\pm$ 4.5	13.4 $\pm$ 2.4	39.8 $\pm$ 2.5	37.2 $\pm$ 5.2	34.8 $\pm$ 7.6	33.3 $\pm$ 10
	Cr(VI)		14.9 $\pm$ 2.0	15.7 $\pm$ 1.1	16.6 $\pm$ 1.2	16.2 $\pm$ 2.6	36.8 $\pm$ 5.8	34.8 $\pm$ 4.2	34.7 $\pm$ 5.8	39.8 $\pm$ 3.5
Protein content 2 (cytosolic fraction) mg/g w.w.	Cr(III)	24 h	9.7 $\pm$ 1.8	11.9 $\pm$ 0.8	12.3 $\pm$ 1.4	11.8 $\pm$ 0.3	27.8 $\pm$ 2.5	25.5 $\pm$ 3.5	26.9 $\pm$ 4.7	30.4 $\pm$ 1.9
	Cr(VI)		12.6 $\pm$ 1.8	12.9 $\pm$ 1.4	12.9 $\pm$ 1.2	12.6 $\pm$ 1.1	28.2 $\pm$ 5.8	31.5 $\pm$ 5.9	25.7 $\pm$ 2.6	23.6 $\pm$ 5.1
	Cr(III)	96 h	15.1 $\pm$ 0.6	12.4 $\pm$ 1	10.7 $\pm$ 2.3	8.7 $\pm$ 1.4	29.3 $\pm$ 2.4	26.3 $\pm$ 2.8	25.4 $\pm$ 6.4	24.9 $\pm$ 6.8
	Cr(VI)		10 $\pm$ 0.7	10.9 $\pm$ 0.8	11.1 $\pm$ 0.8	11.3 $\pm$ 1.6	25.6 $\pm$ 2.2	24.7 $\pm$ 2.4	24.3 $\pm$ 4	25.8 $\pm$ 2.3
Cholesterol mg/g w.w.	Cr(III)	24 h	1.0 $\pm$ 0.3	0.7 $\pm$ 0.2	0.6 $\pm$ 0.2	0.6 $\pm$ 0.1	1.5 $\pm$ 0.2	1.4 $\pm$ 0.3	1.4 $\pm$ 0.4	1.6 $\pm$ 0.3
	Cr(VI)		0.6 $\pm$ 0.2	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	1.5 $\pm$ 0.3	1.7 $\pm$ 0.2	1.5 $\pm$ 0.1	1.5 $\pm$ 0.2
	Cr(III)	96 h	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.2	0.5 $\pm$ 0.1	1.6 $\pm$ 0.2	1.5 $\pm$ 0.3	1.5 $\pm$ 0.4	1.5 $\pm$ 0.3
	Cr(VI)		0.6 $\pm$ 0.1	0.6 $\pm$ 0.01	0.6 $\pm$ 0.01	0.6 $\pm$ 0.1	1.6 $\pm$ 0.3	1.6 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.2
Triglycerides mg/g w.w.	Cr(III)	24 h	1.3 $\pm$ 0.4	1.2 $\pm$ 0.2	1.1 $\pm$ 0.4	1.2 $\pm$ 0.5	4.5 $\pm$ 0.6	5.2 $\pm$ 1.8	4.1 $\pm$ 0.9	4.7 $\pm$ 0.6
	Cr(VI)		1 $\pm$ 0.5	1.2 $\pm$ 0.2	1 $\pm$ 0.1	0.9 $\pm$ 0.2	3.8 $\pm$ 0.8	6.1 $\pm$ 0.8	6.1 $\pm$ 1	4.5 $\pm$ 1.1
	Cr(III)	96 h	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1 $\pm$ 0.3	0.8 $\pm$ 0.2	5.5 $\pm$ 0.2	5.2 $\pm$ 1.2	4.8 $\pm$ 0.9	4.9 $\pm$ 1.5
	Cr(VI)		0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	0.9 $\pm$ 0.2	5.0 $\pm$ 1.1	4.8 $\pm$ 0.7	4.7 $\pm$ 0.7	4.0 $\pm$ 0.4
ETS mmol O ₂ /g prot/h	Cr(III)	24 h	2.3 $\pm$ 0.1	2.4 $\pm$ 0.3	2.3 $\pm$ 0.3	2.3 $\pm$ 0.2	1.1 $\pm$ 0.2	1.1 $\pm$ 0.1	1.3 $\pm$ 0.2	1.4 $\pm$ 0.2
	Cr(VI)		2.4 $\pm$ 0.1	2.2 $\pm$ 0.1	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1	1.8 $\pm$ 0.4	1.5 $\pm$ 0.2	1.7 $\pm$ 0.2	1.8 $\pm$ 0.4
	Cr(III)	96 h	2.1 $\pm$ 0.1	1.9 $\pm$ 0.2	1.8 $\pm$ 0.4	1.4 $\pm$ 0.3	1.6 $\pm$ 0.1	1.8 $\pm$ 0.2	3.6 $\pm$ 1.4	2.2 $\pm$ 0.4
	Cr(VI)		1.4 $\pm$ 0.1	2.3 $\pm$ 0.1	2.2 $\pm$ 0.2	2.2 $\pm$ 0.3	1.8 $\pm$ 0.3	1.7 $\pm$ 0.2	1.9 $\pm$ 0.3	1.9 $\pm$ 0.3

Biomarker	Exposure (Type)	Exposure (Duration)	Gills				Digestive glands			
			0 $\mu\text{g L}^{-1}$	5 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$	500 $\mu\text{g L}^{-1}$	0 $\mu\text{g L}^{-1}$	5 $\mu\text{g L}^{-1}$	50 $\mu\text{g L}^{-1}$	500 $\mu\text{g L}^{-1}$
LOOH $\mu\text{mol/g prot}$	Cr(III)	24 h	41.3 $\pm$ 2.5	37.7 $\pm$ 3.3	33.4 $\pm$ 4.5	30.4 $\pm$ 4.9	27.0 $\pm$ 2.5	30.2 $\pm$ 2.0	24.9 $\pm$ 4.4	22.9 $\pm$ 1.1
	Cr(VI)		34.4 $\pm$ 3.3	35.5 $\pm$ 2.5	37.1 $\pm$ 4.4	36.3 $\pm$ 2.9	24.9 $\pm$ 3.0	20.9 $\pm$ 1.5	23.8 $\pm$ 2.1	24.3 $\pm$ 4.8
	Cr(III)	96 h	33.1 $\pm$ 2.1	38.7 $\pm$ 2.0	42.0 $\pm$ 10.6	37.0 $\pm$ 3.1	21.0 $\pm$ 1.8	21.4 $\pm$ 1.6	24.0 $\pm$ 2.3	27.2 $\pm$ 5.7
	Cr(VI)		31.2 $\pm$ 2.9	40.7 $\pm$ 3.6	38.0 $\pm$ 2.8	39.4 $\pm$ 2.2	26.5 $\pm$ 2.7	24.8 $\pm$ 3.6	26.7 $\pm$ 2.1	21.0 $\pm$ 1.0
ACP $\mu\text{mol/g prot/h}$	Cr(III)	24 h	107 $\pm$ 17	116 $\pm$ 9	107 $\pm$ 16	108 $\pm$ 10	81 $\pm$ 37	89 $\pm$ 28	120 $\pm$ 41	99 $\pm$ 27
	Cr(VI)		117 $\pm$ 7	113 $\pm$ 8	113 $\pm$ 8	104 $\pm$ 6	132 $\pm$ 47	124 $\pm$ 15	132 $\pm$ 4	125 $\pm$ 23
	Cr(III)	96 h	115 $\pm$ 5	106 $\pm$ 7	106 $\pm$ 7	92 $\pm$ 9	103 $\pm$ 31	111 $\pm$ 33	104 $\pm$ 38	113 $\pm$ 29
	Cr(VI)		95 $\pm$ 7	102 $\pm$ 8	105 $\pm$ 1	95 $\pm$ 6	84 $\pm$ 24	87 $\pm$ 16	122 $\pm$ 37	88 $\pm$ 17
LDH $\mu\text{M NADPH/g prot/h}$	Cr(III)	24 h	75 $\pm$ 13	100 $\pm$ 12	106 $\pm$ 32	108 $\pm$ 33	129 $\pm$ 27	116 $\pm$ 30	157 $\pm$ 78	121 $\pm$ 50
	Cr(VI)		114 $\pm$ 13	108 $\pm$ 17	98 $\pm$ 18	103 $\pm$ 18	145 $\pm$ 39	133 $\pm$ 32	107 $\pm$ 48	175 $\pm$ 40
	Cr(III)	96 h	96 $\pm$ 10	90 $\pm$ 12	111 $\pm$ 58	183 $\pm$ 32	129 $\pm$ 71	94 $\pm$ 73	130 $\pm$ 77	93 $\pm$ 34
	Cr(VI)		179 $\pm$ 32	85 $\pm$ 3	75 $\pm$ 16	120 $\pm$ 16	90 $\pm$ 27	138 $\pm$ 50	156 $\pm$ 79	112 $\pm$ 35
GST $\mu\text{mol CDNB/g prot/min}$	Cr(III)	24 h	391 $\pm$ 46	397 $\pm$ 67	384 $\pm$ 170	367 $\pm$ 114	708 $\pm$ 190	887 $\pm$ 208	955 $\pm$ 611	583 $\pm$ 146
	Cr(VI)		440 $\pm$ 44	366 $\pm$ 25	301 $\pm$ 47	303 $\pm$ 35	710 $\pm$ 221	632 $\pm$ 144	746.7 $\pm$ 43	1068 $\pm$ 205
	Cr(III)	96 h	249 $\pm$ 11	277 $\pm$ 75	387 $\pm$ 106	329 $\pm$ 65	607 $\pm$ 173	643 $\pm$ 205	612 $\pm$ 260	605 $\pm$ 80
	Cr(VI)		348 $\pm$ 44	597 $\pm$ 87	443 $\pm$ 57	441 $\pm$ 165	558 $\pm$ 275	701 $\pm$ 173	841 $\pm$ 337	695 $\pm$ 174
CSP3 $\mu\text{mol pNA/g prot/h}$	Cr(III)	24 h	6.1 $\pm$ 2.0	10.2 $\pm$ 5	8.0 $\pm$ 5.5	18.7 $\pm$ 6.5	4.9 $\pm$ 3.3	6.3 $\pm$ 4.2	7.6 $\pm$ 7.5	7.1 $\pm$ 5.7
	Cr(VI)		4.0 $\pm$ 0.9	6.3 $\pm$ 0.5	5.9 $\pm$ 1.4	10.5 $\pm$ 5.2	2.9 $\pm$ 1.6	10 $\pm$ 5.2	8.5 $\pm$ 6.2	19 $\pm$ 8.4
	Cr(III)	96 h	7.2 $\pm$ 1.7	6.3 $\pm$ 2.7	4.1 $\pm$ 0.8	3.0 $\pm$ 1.2	9.4 $\pm$ 7.9	6.8 $\pm$ 7.7	4.8 $\pm$ 7.1	1.7 $\pm$ 1.3
	Cr(VI)		3.1 $\pm$ 0.6	4.7 $\pm$ 1.1	5.3 $\pm$ 1.8	9.5 $\pm$ 3.5	1.2 $\pm$ 2.7	2.6 $\pm$ 2.6	5.8 $\pm$ 6.4	1.2 $\pm$ 1
Catalase (CAT) $\text{mmole H}_2\text{O}_2/\text{g prot/min}$	Cr(III)	24 h	51 $\pm$ 5.8	47 $\pm$ 2.8	42 $\pm$ 7.7	43 $\pm$ 5.5	72 $\pm$ 15	89 $\pm$ 25	115 $\pm$ 39 ^{\$}	105 $\pm$ 63
	Cr(VI)		41 $\pm$ 1.3	40 $\pm$ 6.9	37 $\pm$ 4.2	39 $\pm$ 3.5	61 $\pm$ 46	82 $\pm$ 16	78 $\pm$ 43	99 $\pm$ 39
	Cr(III)	96 h	41 $\pm$ 4.2	43 $\pm$ 2.7	42 $\pm$ 17	34 $\pm$ 11	63 $\pm$ 22	52 $\pm$ 22	50 $\pm$ 27 ^{\$}	62 $\pm$ 36
	Cr(VI)		36 $\pm$ 13	49 $\pm$ 8.9	45 $\pm$ 4.3	46 $\pm$ 9.3	39 $\pm$ 33	67 $\pm$ 37	67 $\pm$ 21	73 $\pm$ 28

Biomarker	Exposure (Type)	Exposure (Duration)	Gills				Digestive glands			
			0 µg L ⁻¹	5 µg L ⁻¹	50 µg L ⁻¹	500 µg L ⁻¹	0 µg L ⁻¹	5 µg L ⁻¹	50 µg L ⁻¹	500 µg L ⁻¹
TAC µmolTeq/g prot/min	Cr(III)	24 h	-	-	-	-	25 ± 3.7	25 ± 3.9	29 ± 8.6	18 ± 3.0
	Cr(VI)		-	-	-	-	23 ± 6.7	27 ± 3.9	27 ± 3.7	37 ± 5.3
	Cr(III)	96 h	-	-	-	-	27 ± 9.6	24 ± 9.7	22 ± 8.8	19 ± 5.0
	Cr(VI)		-	-	-	-	14 ± 5.6	22 ± 4.6	27 ± 9.7	20 ± 3.1
GPx µmolTeq/g prot/min	Cr(III)	24 h	-	-	-	-	-	-	-	-
	Cr(VI)		-	-	-	-	-	-	-	-
	Cr(III)	96 h	-	-	-	-	-	-	-	-
	Cr(VI)		-	-	-	-	-	-	-	-

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982 Table S8. Activities of cellular biomarkers (arithmetic means $\pm$ 1 standard deviation; n =3) measured in gills and digestive glands of the
983 freshwater bivalve *Corbicula fluminea* in exp# 2. CAT and GPx could not be quantified and were excluded from the table. w.w., wet weight.

Biomarker	Exposure (type)	Gills	Digestive glands	Biomarker	Exposure (type)	Gills	Digestive glands
Protein content (homogenate) mg/g w.w.	Control	11.6 $\pm$ 3.8	25.6 $\pm$ 4	ACP μ mol/g prot/h	Control	89 $\pm$ 18	85 $\pm$ 15
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	14.9 $\pm$ 2.3	28.9 $\pm$ 4.2		⁵⁰ Cr(III) + ⁵³ Cr(VI)	89 $\pm$ 17	76 $\pm$ 20
	Cr(III)	15.6 $\pm$ 2.2	29.6 $\pm$ 1.8		Cr(III)	78 $\pm$ 14	80 $\pm$ 13
	Cr(VI)	11.5 $\pm$ 2.5	32.5 $\pm$ 6		Cr(VI)	77 $\pm$ 18	83 $\pm$ 22
Protein content (cytosolic fraction) mg/g w.w.	Control	8.6 $\pm$ 0.8	19.7 $\pm$ 2.7	LDH μ mol NADPH/g prot/h	Control	135 $\pm$ 35	182 $\pm$ 88
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	10 $\pm$ 1.8	20.7 $\pm$ 2.4		⁵⁰ Cr(III) + ⁵³ Cr(VI)	107 $\pm$ 35	121 $\pm$ 35
	Cr(III)	9.9 $\pm$ 1.7	21.7 $\pm$ 0.7		Cr(III)	95 $\pm$ 29	137 $\pm$ 65
	Cr(VI)	9.6 $\pm$ 0.3	22 $\pm$ 3.8		Cr(VI)	110 $\pm$ 59	170 $\pm$ 121
Cholesterol mg/g w.w.	Control	0.48 $\pm$ 0.16	1.03 $\pm$ 0.25	TAC μ mol Teq/g prot/min	Control	-	31.3 $\pm$ 6.5
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	0.61 $\pm$ 0.1	1.23 $\pm$ 0.24		⁵⁰ Cr(III) + ⁵³ Cr(VI)	-	25.5 $\pm$ 3.9
	Cr(III)	0.61 $\pm$ 0.1	1.20 $\pm$ 0.17		Cr(III)	-	28.9 $\pm$ 5.2
	Cr(VI)	0.48 $\pm$ 0.12	1.29 $\pm$ 0.34		Cr(VI)	-	53.9 $\pm$ 40.7
Triglycerides mg/g w.w.	Control	0.45 $\pm$ 0.24	3.59 $\pm$ 1.38	GST μ mol CDNB/ g prot/min	Control	343 $\pm$ 126	982 $\pm$ 345
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	0.45 $\pm$ 0.1	4.22 $\pm$ 1.53		⁵⁰ Cr(III) + ⁵³ Cr(VI)	428 $\pm$ 178	1001 $\pm$ 333
	Cr(III)	0.58 $\pm$ 0.1	3.78 $\pm$ 0.83		Cr(III)	415 $\pm$ 77	972 $\pm$ 184
	Cr(VI)	0.43 $\pm$ 0.2	4.08 $\pm$ 1.28		Cr(VI)	461 $\pm$ 126	949 $\pm$ 173
ETS mmol O ₂ /g prot/h	Control	2.8 $\pm$ 0.1	1.8 $\pm$ 0.1	CSP3 μ mol pNA/g prot/h	Control	13 $\pm$ 5.9	7 $\pm$ 3.6
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	3.1 $\pm$ 0.1	1.8 $\pm$ 0.4		⁵⁰ Cr(III) + ⁵³ Cr(VI)	13.7 $\pm$ 1.6	3.8 $\pm$ 2.6
	Cr(III)	2.8 $\pm$ 0.2	1.7 $\pm$ 0.2		Cr(III)	19.8 $\pm$ 5.8	5.5 $\pm$ 3.6
	Cr(VI)	2.9 $\pm$ 0.3	1.6 $\pm$ 0.3		Cr(VI)	4.3 $\pm$ 1.7	5.8 $\pm$ 4.7
LOOH μ mol/g prot	Control	64.3 $\pm$ 9.7	22.4 $\pm$ 0.9				
	⁵⁰ Cr(III) + ⁵³ Cr(VI)	55.9 $\pm$ 5.8	23.2 $\pm$ 1.3				
	Cr(III)	61.9 $\pm$ 3	22.6 $\pm$ 1.4				
	Cr(VI)	57.4 $\pm$ 1.8	24.6 $\pm$ 3.1				

984 Table S9. Regression analysis of the biomarkers' expression (after Log₁₀ transformation) as a function of the different explanatory
 985 variables: exposure duration (D), Cr redox form (Cr), exposure concentration (Co), tissue (T) and their interactions (*). A probability
 986 threshold of 0.01 was used for the construction of all fitted models. r²: coefficient of determination.

Experiment 1					Experiment 2		
Biomarker	Tissue	Fitted model Log10 (biomarker)	F-test P-value	r ²	Fitted model Log10 (biomarker)	F-test P-value	r ²
Chol	G		0.209		- 0.104 + 0.171 T	< 10 ⁻⁴	0.772
	DG		0.881				
LDH	G		0.010			0.866	
	DG		0.559				
TG	G		0.107		0.118 + 0.459 T	< 10 ⁻⁴	0.927
	DG		0.510				
Prot1	G		0.059		1.287 + 0.173 T	< 10 ⁻⁴	0.800
	DG	1.563 + 1.096·10 ⁻⁴ D*Cr*Co	0.003	0.129			
Prot2	G	1.061 - 0.035 D*Cr - 1.008·10 ⁻⁴ D*Co + + 1.195·10 ⁻⁴ D*Cr*Co	< 10 ⁻⁴	0.302	1.148 + 0.173 T	< 10 ⁻⁴	0.924
	DG		0.180				
GST	G	2.558 + 0.049 D*Cr	0.001	0.161	2.786 + 0.191 T	< 10 ⁻⁴	0.703
	DG		0.055				
LOOH	G		0.114		1.570 - 0.205 T	< 10 ⁻⁴	0.968
	DG	1.384 + 0.038 D*Cr - 1.416·10 ⁻⁴ D*Cr*Co	< 10 ⁻⁴	0.320			
ACP	G	2.036 - 0.018 D - 0.753·10 ⁻⁴ Co	< 10 ⁻⁴	0.290		0.962	
	DG	2.009 - 0.046 D*Cr	0.006	0.118			
CSP3	G	0.725 - 0.069 Cr + 3.724·10 ⁻⁴ Co - 4.168·10 ⁻⁴ D*Co + + 3.911·10 ⁻⁴ D*Cr*Co	< 10 ⁻⁴	0.460	0.848 - 0.180T	0.005	0.310
	DG	0.550 - 9.121·10 ⁻⁴ D*Co	< 10 ⁻³	0.175			
ETS	G	0.316 - 0.042 D + 0.974·10 ⁻⁴ Cr*Co + 1.094·10 ⁻⁴ D*Cr*Co	< 10 ⁻⁴	0.439	0.347 - 0.115 T	< 10 ⁻⁴	0.844
	DG	0.224 + 0.072 D - 0.051 D*Cr	< 10 ⁻⁴	0.440			

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Table S10. Regression analysis of the mRNA expression (after Log₁₀ transformation) as a function of the Cr redox form (Cr) and the exposure concentration (Co) after 96 h of exposure (experiment 1). A probability threshold of 0.01 was used for the construction of all fitted models. r²: coefficient of determination. G, gills; DG, digestive glands.

mRNA	Tissue	Fitted model	F-test	
		Log ₁₀ (mRNA)	P-value	r ²
GST pi	G	0.207 - 2.671 10 ⁻⁴ Cr*Co ^(a)	0.012	0.255
	DG		0.706	
GPx	G	Not performed ^(b)		
	DG		0.405	
CAT	G	-1.482 - 6.294 10 ⁻⁴ Cr*Co	< 10 ⁻³	0.475
	DG		0.183	
CuZn SOD	G	-0.516 - 10.148 10 ⁻⁴ Cr*Co	< 0.01	0.276
	DG		0.090	
Mn SOD	G	-2.146 - 4.477 10 ⁻⁴ Cr*Co	< 10 ⁻³	0.472
	DG		0.201	
TR	G		0.116	
	DG	-1.407 + 0.074 Cr + 3.966 10 ⁻⁴ Co	0.002	0.439
MT	G	0.423 - 3.556 10 ⁻⁴ Cr*Co	0.016	0.237
	DG	0.801 - 2.862 10 ⁻⁴ Co	0.011	0.259
HSP70	G	0.458 - 4.447 10 ⁻⁴ Cr*Co	<0.01	0.268
	DG	0.120 + 0.091 Cr + 5.077 10 ⁻⁴ Co	<10 ⁻⁴	0.668

(a) See also table S2 for the coding of Cr redox forms. The coding for Cr(III) is '-1', meaning that mRNA expression increases with concentration because the minus sign of the coefficient for 'Cr*Co' and the minus sign for Cr(III) factor give a + sign. The opposite situation occurs for Cr(VI).

(b) Insufficient GPx level.