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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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25 **Abstract**

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

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

49 **Highlights**

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

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

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

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

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

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73 **1. Introduction**

74 Chromium is a widespread environmental contaminant due to its use in several industrial
75 processes (WHO, 2009; 2013). In natural environments, Cr mostly occurs in two oxidation states,
76 Cr(III) and Cr(VI), that have markedly different environmental and biological behaviour.
77 Chromium(VI) is a recognized human carcinogen and has a high environmental mobility,
78 bioavailability and (eco)toxicity. These biogeochemical properties follow from chromate ions
79 being the most common chemical species of Cr(VI) (Gorny et al., 2016). Chromate ions are
80 structural analogues of sulphates and can easily cross biological membranes via facilitated
81 transport (Viti et al., 2014). Once inside the cell, Cr(VI) undergoes rapid reduction to Cr(III). The
82 reduction reactions cause oxidative stress damage and produce intracellular Cr(III) that can
83 further react with nucleic acids causing mutagenic and genotoxic effects (Kortenkamp et al.,
84 1991; Sawicka et al., 2021; Zhitkovich, 2005). Chromate ions also show limited reactivity
85 towards environmental particles, which usually bear a net negative charge at pH values above 4
86 (Warren and Haack, 2001). Chromate ions can therefore undergo long-distance transport and
87 migrate into groundwater (Guo et al., 2020; Izbicki et al., 2015; Oze et al., 2007).

88 Chromium(III) is putatively less bioavailable and (eco)toxic, but increasing evidence suggests
89 that it can also elicit adverse effects in humans as well as in aquatic and terrestrial organisms
90 (Aharchaou et al., 2018; Park, 2020; Tsuchiyama et al., 2020). Mechanism of Cr(III) uptake are
91 less studied than those for Cr(VI) and may involve Cr(III)-transferrin complexes, endocytosis of
92 Cr(III)-bearing particles or uptake of hydrophobic Cr(III) complexes (Beyersmann and Hartwig,
93 2008; Viti et al., 2014). Effects of Cr(III) have also been reported on the structure and
94 permeability of biological membranes (Filice et al., 2019; Suwalsky et al., 2008). Finally, Cr(III)
95 is particle reactive and tends to adsorb onto suspended particulate matter and colloids eventually
96 accumulating in bed sediments (Dominik et al., 2007; Gustafsson et al., 2014; Liao et al., 2020).

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

112 In principle, it is relatively easy to prepare laboratory exposure media containing both Cr
113 redox forms. On the other hand, it remains far from straightforward to understand the relative
114 contribution of Cr(III) and Cr(VI) to the total Cr body burden and biological responses of the
115 exposed organisms. In aqueous matrices, including ecotoxicological test media, the redox
116 speciation of Cr can be determined using ion Chromatography ICP-MS (Aharchaou et al., 2018;
117 Markiewicz et al., 2015; Seby et al., 2003). However, the redox speciation of Cr in the exposure
118 medium will not be retained in the test organisms, because Cr(VI) is rapidly reduced to Cr(III) in
119 the intracellular environment (Beyersmann and Hartwig, 2008; Sawicka et al., 2021; Zhitkovich,
120 2005). Tracking the fate of Cr(III) and Cr(VI) from exposure solution to biological tissues is

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

127 Chromium has four naturally occurring stable isotopes ($^{50}\text{Cr} = 4.345\%$, $^{52}\text{Cr} = 83.789\%$, ^{53}Cr
128 $= 9.501\%$ and $^{54}\text{Cr} = 2.365\%$) that are widely studied in relation to cosmochemical, geochemical
129 and environmental processes (Cole et al., 2017; Rodríguez-González et al., 2005; Wei et al.,
130 2020). Ecotoxicological studies on Cr ecotoxicity usually employ commercially available Cr(III)
131 and Cr(VI) salts that contain all four isotopes at the corresponding natural abundances. However,
132 materials enriched in one specific isotope and in a specific redox form are also available for
133 purchase (Martone et al., 2013; Novotnik et al., 2012). In the absence of redox interconversions
134 between Cr(III) and Cr(VI) in the exposure medium, the fate of each isotope (i.e., redox form)
135 can be followed from the exposure solution to the biological tissue via a simple ICP-MS analysis
136 at the right m/z value. In the present study, specimen of the freshwater bivalve *Corbicula*
137 *fluminea* were exposed to solutions amended with Cr(III) or Cr(VI) salts having natural isotopic
138 abundances, or with a mixture of $^{50}\text{Cr(III)} + ^{53}\text{Cr(VI)}$. *Corbicula fluminea* is a widespread filter-
139 feeder that is largely used as a sentinel organism for freshwater biomonitoring of metal
140 accumulation and sublethal effects (de Oliveira and dos Reis Martinez, 2014; Marie et al., 2006;
141 Pereto et al., 2020). The specific objectives of the present study were to determine: (1) the effect
142 of the individual Cr redox forms and of the $^{50}\text{Cr(III)} + ^{53}\text{Cr(VI)}$ mixture on the expression of
143 several biomarkers and mRNA, and (2) the bioaccumulation of Cr in the bivalves' gills and
144 digestive glands.

145
146 **2. Materials and methods**
147 *2.1. Organism collection and acclimation*
148 Individuals of *Corbicula fluminea* (size 2–4 cm) were collected in the Moselle River (France) at
149 GPS coordinates 49°07'06.5'' N, 6°09'39.0'' E and kept until the end of sampling in unfiltered
150 site water (pH = 8.0, conductivity = 1823 $\mu\text{S cm}^{-1}$, oxygen saturation = 103%). Upon return to the
151 laboratory, organisms were kept in aerated water at 15 ± 0.1 °C and acclimated to the test
152 medium (Cristaline® mineral water, spring Aurèle, see composition in Table S1) by replacing 1/3
153 site water with spring water at 24 h intervals over four days (Potet et al., 2016). The viability of
154 organisms was checked at each medium renewal by testing their ability to close the valves after a
155 gentle pressure on the ligament.

156
157 *2.2. Exposure set-up*
158 A preliminary test was performed to establish suitable exposure conditions for the measurement
159 of sublethal effects and bioaccumulation of Cr. After acclimation (section 2.1), twelve individuals
160 of *C. fluminea* were transferred to 3 L aquaria containing 1.5 L of Cristaline® mineral water
161 amended with 5, 50, 500, 1000, 2000 and 5000 $\mu\text{g L}^{-1}$ of Cr(III) or Cr(VI). Trivalent Cr was
162 added as $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (Alfa Aesar, purity 99.5 % min, crystalline, CAS 10060-12-5) and
163 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
164 isotopic abundances (NIA). Exposure medium was continuously aerated by bubbling ambient air
165 in each aquarium. Organisms were kept at 15 °C under artificial daylight (800–1000 Lux) and a
166 12 h light – 12 h dark photoperiod. Organisms were not fed during the experiments. No mortality
167 was observed across the entire concentration range for neither Cr(III) nor Cr(VI) (data not

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

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

178 A second experiment (exp #2) examined the relationships between biomarker expression and
179 Cr bioaccumulation in the gills and digestive glands of *C. fluminea*. Organisms were exposed to:
180 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)
181 + 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
182 Isotope Technologies – AIT, Pittsburg, PA, USA: batch # SH1332, Lot # CR12222014JC; PN-
183 20529, isotopic enrichment 97.7 % for $^{53}\text{Cr(VI)}$ and batch # 030129-04-A, Lot # Cr04272010A;
184 PN-2; isotopic enrichment 97.3 % for $^{50}\text{Cr(III)}$. All tests lasted for 96 h and were carried out
185 under the same conditions as described above with 12 individuals for each scenario plus two
186 control batches of 12 individuals each. The selected concentrations were considered a good
187 compromise between practical constraints (notably the availability of IES and analytical detection
188 in biological tissues) and environmental relevance for contaminated environments (Gunkel-
189 Grillon et al., 2014; Hayzoun et al., 2015).

190

191

192 2.3. Chromium measurements in aqueous and biological matrices

193 In exp #1, total and total filterable Cr concentrations were measured at t = 0, 2, 4, 6 and 24 h (n =
194 1 to 3). Filtration was carried out using 0.45 µm membrane filters (Merck Millipore, Millex
195 33mm, PVDF, reference # SLHV033NB). For experiments lasting 96 h, measurements were
196 carried out over a single renewal period of 24 h and considered representative for all exposure
197 periods. Samples were acidified at 1 % v/v with concentrated nitric acid (65%, VWR, Suprapur)
198 and analysed by Flame Atomic Absorption Spectroscopy (FAAS; Perkin-Elmer AAnalyst 100) or
199 Graphite Furnace Flame Atomic Absorption (GFAAS; Varian spectra 880Z+). Measurement
200 accuracy was verified using certified reference materials SPS-SW1 (2.00 ± 0.02 µg L⁻¹) and
201 WW1 (200 ± 1 µg L⁻¹), both from Spectrapure Standards (Oslo, Norway).

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

209 $^{50}\text{Cr}_{\text{corrected}} = (^{50}\text{Cr}_{\text{ICP-MSsoftware}} \cdot 4.345) \cdot (100/\text{baseline drift})$ (Eq. 1)

210 $^{53}\text{Cr}_{\text{corrected}} = (^{53}\text{Cr}_{\text{ICP-MSsoftware}} \cdot 9.500) \cdot (100/\text{baseline drift})$ (Eq. 2)

211 $^{52}\text{Cr}_{\text{corrected}} = (^{52}\text{Cr}_{\text{ICP-MSsoftware}}/\text{baseline drift})$ (Eq. 3)

212 Where:

213 $^{xx}\text{Cr}_{\text{corrected}}$ are the Cr concentrations corrected for isotopic abundances and for the ICP-MS
214 baseline drift (see below);

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

221 Chromium levels in gills and digestive glands of *C. fluminea* were assayed by ICP-MS at m/z
222 50, 52 and 53 using KED mode after mineralization with 0.5 mL of concentrated nitric acid (65%
223 v/v, Suprapur, VWR) and 0.1 mL of H_2O_2 (30% v/v, Suprapur, VWR). Mineralization was
224 carried out in acid-washed polystyrene tubes placed in a stove at 65 °C overnight. Raw analytical
225 results were corrected as necessary using equations 1 to 3. Gills and digestive glands were
226 recovered by immediately dissecting exposed organisms at the end of the exposure period.
227 Tissues were frozen at -20 °C and then freeze-dried over 24 h (CRIOS-80, Cryotech). The
228 accuracy of the mineralization procedure was checked using the reference material TORT-2
229 (lobster hepatopancreas) from the National Research Council of Canada (NRC-CNRC). The
230 certified value for total Cr was $0.77 \pm 0.15 \text{ mg kg}^{-1}$ (uncertainty expressed as 95% confidence
231 limits). The ICP-MS detection and quantification limits were calculated as three and ten times the
232 standard deviation of replicate analytical blanks ($n = 14$).

233

234 *2.4. Biomarker expression*

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

239 determined. Tissues were then transferred to microtubes (2 mL) and manually crushed in 50mM
240 phosphate buffer (98% v/v, pH = 7.6) supplemented with 1% v/v of phenylmethylsulfonyl
241 fluoride (PMSF) and 1% v/v of a 1 mM mixture of L-serine and borate to inhibit proteases
242 (Garaud et al., 2015; Sroda and Cossu-Leguille, 2011). The quantity of added buffer was adjusted
243 to the mass of tissue based on a ratio of 4 mL of buffer to 500 mg of tissue (wet weight). Samples
244 were then centrifuged for 5 min (250 g) at 4 °C. Part of the resulting tissue homogenate was used
245 as such for biomarker analysis. A second aliquot of homogenate was again centrifuged for 20 min
246 (1000 g) at 4 °C. The resulting supernatant was subject to a final centrifugation (50 min, 20000 g,
247 4 °C) to recover the cytosolic fraction for the measurements of enzymatic activities.

248 Tissue homogenates were used to assay markers of cellular damage (LOOH, *Lipid*
249 *Hydroperoxide*; ACP, *Acid phosphatase*), the status of energy reserves (Prot1, *proteins*; TG,
250 *triglycerides*), Cholesterol (Chol.), and the activity of the mitochondrial Electron Transport
251 Systems (ETS). Other markers of oxidative stress (TAC, *Total antioxidant capacity*; GPx-tot,
252 *Glutathione peroxidase*; GST, *Glutathione S-transferase*; CAT, *Catalase*), cellular damage (CSP-
253 3, *Caspase-3*) and energy reserves (Prot2, *proteins*; LDH, *Lactate dehydrogenase*) were
254 measured in the cytosolic fraction. All biomarkers were analysed using an automated
255 spectrophotometer (Konelab 20 XTi, Thermo Scientific) according to the methods described in
256 Garaud et al. (2015), Bertrand et al. (2016) and references therein.

257

258 2.5. RNA extraction and RT-qPCR analysis

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

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

268 RT-qPCR was performed on 8 detoxification genes (GST-pi, CAT, Mn SOD, CuZn SOD, TR,
269 MT, HSP70 and Se-GPX) and PS3 as reference gene using the methodologies described in
270 Koehle-Divo et al. (2019) and the sequences reported in Table S2. cDNA was reverse-transcribed
271 from total RNA (900 ng) using the Superscript IV Reverse Transcriptase with oligo(dT)₁₈
272 according to the manufacturer recommendations (Invitrogen, Carlsbad, CA, USA). Real-time
273 PCR was performed on a StepOnePlus PCR system using the Fast SYBR® green master mix
274 (Applied Biosystem®, CA, USA). Amplifications were performed with 50 ng of template, or
275 water (no template control), or 50 ng total RNA (no RT control) using 200 to 300 nM of primers
276 in a final volume of 20 μ L (Koehle-Divo et al., 2019). The cycling conditions were 20 s at 95°C,
277 followed by 40 cycles of 10 s at 95°C (except for CuZn SOD, HSP70, PS3 and GST-pi: 3 s at
278 95°C) and 30 s at 60° C. A melting curve analysis (65° C–95° C) was performed to ensure the
279 absence of artefacts and the expression of the genes of interest was normalized to that of PS3
280 using the $2^{-\Delta\Delta C_t}$ method.

281

282 *2.6. Statistical methods and data treatment*

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

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

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

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

301 (Eq. 4a)

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

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

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

307 (Eq. 4b)

308

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

312

$$\begin{aligned} 313 \text{Log}_{10} (\text{biomarker}) = & \beta_0 + \beta_1 T + \beta_2 \text{Cr3} + \beta_3 \text{Cr6} + \beta_{12} T * \text{Cr3} + \beta_{13} T * \text{Cr6} + \beta_{23} \text{Cr3} * \text{Cr6} + \\ 314 & \beta_{123} T * \text{Cr3} * \text{Cr6} \end{aligned} \quad (\text{Eq.5a})$$

315

$$\begin{aligned} 316 \text{Log}_{10} (\text{total Cr}) = & \beta_0 + \beta_1 T + \beta_2 \text{Cr3} + \beta_3 \text{Cr6} + \beta_{12} T * \text{Cr3} + \beta_{13} T * \text{Cr6} + \beta_{23} \text{Cr3} * \text{Cr6} + \\ 317 & \beta_{123} T * \text{Cr3} * \text{Cr6} \end{aligned} \quad (\text{Eq.5b})$$

318

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

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

324

$$325 \quad BCF = \frac{C_{biota}}{C_{water}} \quad (\text{Eq. 6})$$

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

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

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

333

334

335 **3. Results and discussion**

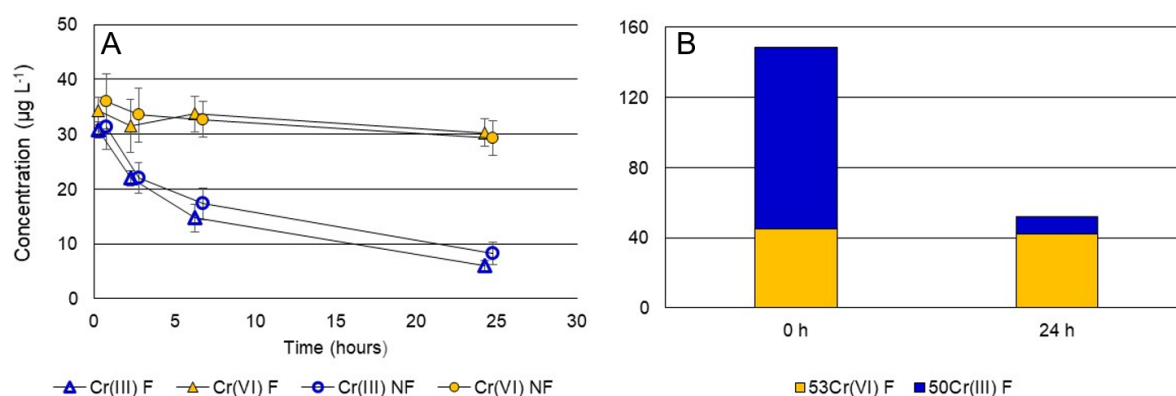
336 *3.1. Chromium concentrations and behaviour in test solutions*

337 Figure of merits for FAAS and GFAAS were the same as previously reported (Aharchaou et
338 al., 2018) and analytical blank values were below detection limits. Measured Cr concentration in
339 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
340 (101 % accuracy). Detection and quantification limits for analyses by ICP-MS were: ^{50}Cr = 0.074
341 and 0.247 $\mu\text{g L}^{-1}$, ^{52}Cr = 0.037 and 0.124 $\mu\text{g L}^{-1}$, ^{53}Cr 0.034 and 0.115 $\mu\text{g L}^{-1}$. Analytical blank
342 values were below detection limits for ^{50}Cr and for ^{53}Cr at t = 0; between detection and
343 quantification limits for ^{53}Cr at t = 24 and measurable for ^{52}Cr . Results for experiments carried
344 out using Cr salts with NIA (for which ^{52}Cr was used) were therefore corrected for the
345 corresponding average analytical blanks. A 200 $\mu\text{g L}^{-1}$ standard solution was repeatedly analysed
346 (n = 5) during the ICP-MS run with average values of $238 \pm 6 \mu\text{g L}^{-1}$ at m/z = 52, $197 \pm 4 \mu\text{g L}^{-1}$
347 at m/z = 50, and $196 \pm 6 \mu\text{g L}^{-1}$ at m/z = 53.

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

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

361



362
 363 Figure 1. Panel A: temporal evolution of total (NF) and filterable (F, $< 0.45 \mu\text{m}$) Cr
 364 concentrations in solutions amended with $50 \mu\text{g L}^{-1}$ of either Cr(III) or Cr(VI) with natural
 365 isotopic abundances (arithmetic means ± 1 standard deviation, $n=3$ for each point). Symbols for
 366 total and filterable values have been slightly offset for representation purposes, but samples for
 367 measurements were collected at the same time. Panel B: filterable Cr concentrations measured in
 368 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
 369 isotopically enriched solutions. See Table S4 for tabulated data.

370
 371 The decrease in total and filterable chromium concentrations in all solutions amended Cr(III)
 372 agrees with the known tendency of Cr(III) to form insoluble Cr(OH)_3 species at circumneutral pH
 373 (Rai et al., 1989). Speciation calculation predicted that approx. 81 % and 98 % of the added

374 Cr(III) would precipitate for initial concentrations of 50 and 500 $\mu\text{g L}^{-1}$. According to
375 VisualMinteq calculations, the theoretical maximum soluble Cr(III) concentration in Cristaline®
376 water is about 10 $\mu\text{g L}^{-1}$ at a pH of 7.4 (measured at the source); in reasonable agreement with
377 experimentally measured Cr concentrations at 24 hours (Fig. 1 and Table S4). On the other hand,
378 the model does not predict the formation of any precipitate for added Cr(III) concentrations of 5
379 $\mu\text{g L}^{-1}$, in disagreement with the experimental results (Table S4).

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

389

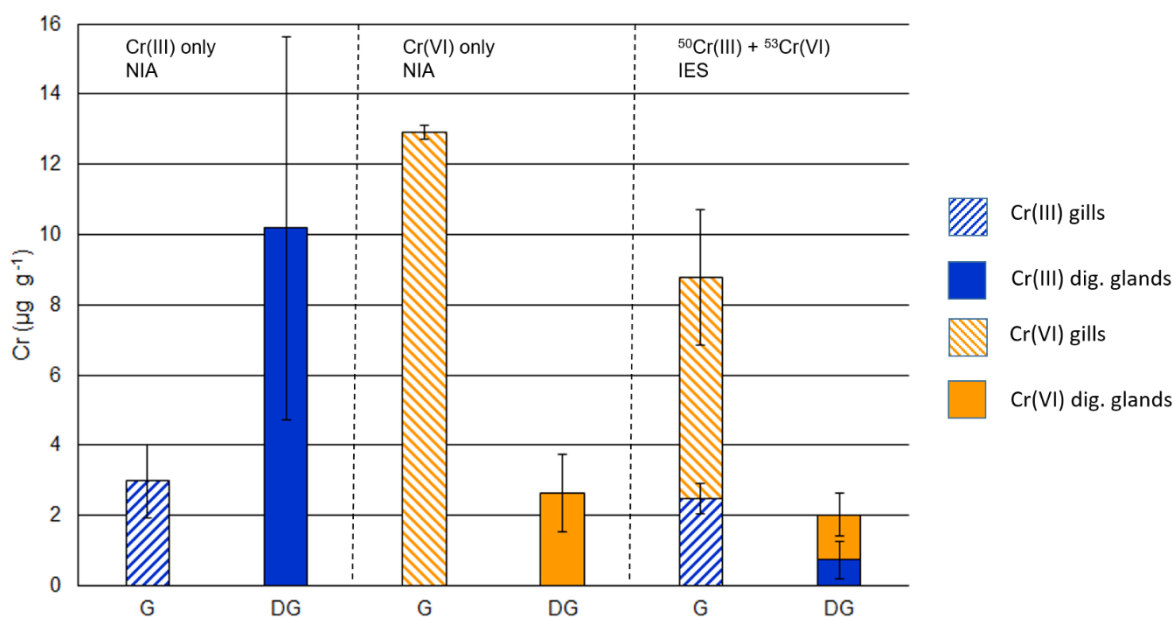
390 3.2. Chromium bioaccumulation and bioavailability

391 Chromium levels in control organisms were above the analytical quantification limits (see
392 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
393 glands (DG). Results for Cr accumulation were therefore corrected for the corresponding blank
394 values in specimen exposed to solutions prepared using Cr salts with NIA (for which
395 measurements were performed at m/z 52). No blank corrections were necessary for specimen
396 exposed to $^{50}\text{Cr(III)}$ and $^{53}\text{Cr(VI)}$ using IES. Duplicate analysis of the reference material TORT2

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

399 For specimen exposed to individual Cr redox forms, Cr(III) accumulation was higher in DG
400 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)
401 (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)
402 mixture, Cr accumulation was higher in G than in DG for both redox forms (Mann-Whitney-
403 Wilcoxon test; $P=0.029$).

404



405
406 Figure 2. Arithmetic means of Cr concentrations accumulated in gills (hatched bars) and
407 digestive glands (full bars) of individuals of *Corbicula fluminea* exposed for 96 hours to solutions
408 amended with either Cr(III) or Cr(VI) salts having natural isotopic abundances or to a solution
409 simultaneously amended with a mixture of ⁵⁰Cr(III) and ⁵³Cr(VI) using isotopically enriched
410 solutions (see Table S4, exp #2, for actual exposure concentrations in each scenario). Errors are
411 expressed as ± 1 standard deviation. Analytical results for individual samples are given in Table
412 S5. NIA, solutions with Natural Isotopic Abundances; IES, Isotopically Enriched Solutions.

413

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

415

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

418

419 with a high explanatory power and predictive capacity (F test, $P < 10^{-4}$; $r^2 = 0.954$; coefficient of
420 variation of the residuals = 34.11%). Except for the three-way interaction $T*\text{Cr3}*\text{Cr6}$ ($P=0.03$),
421 all factors had a statistically significant effect ($P<0.01$) on the bioaccumulated total Cr
422 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
423 results of the fitted model agreed well with the experimentally measured total Cr concentrations
424 (Table 1).

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

437 specimen exposed to Cr(VI) or Cr(III) solutions prepared using salts with NIA, the coefficient of
438 variation of the measured Cr concentrations obtained at m/z 50, 52 and 53 was between 8 and
439 12%; *i.e.*, not large enough to account for the observed decreases in Cr(III) and Cr(VI)
440 bioaccumulation during mixture exposure.

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

454
455
456 Table 1. Total chromium concentrations (95% confidence intervals are given in brackets)
457 accumulated in gills and digestive glands for each combination of factors in experiment 2 as
458 estimated by the fitted reduced model described in Eq. 7. The corresponding arithmetic means of
459 measured concentrations (in italics) are reported for comparison purposes (see Table S5).
460 Nominal exposure concentrations were 0 or 50 $\mu\text{g L}^{-1}$ except for ‘\$’ that was 100 $\mu\text{g L}^{-1}$ (see

461 section 2.2). Concentrations in organisms exposed to either Cr(III) or Cr(VI) have been corrected
 462 for the corresponding blank values of control organisms, while no blank corrections were
 463 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>	---

464

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

466 [‡] Example of calculation using Eq. 7. Remembering that the actual numerical values of the
 467 factors Cr3, Cr6 and T in Eq. 7 correspond to the codes in Table S3, total Cr accumulation in gills
 468 (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)
 469 (code = -1) leads to the following development for Eq. 7: $\text{Log}_{10}(\text{total Cr}) = 0.405 - [0.164 \times (-1)] +$
 470 $[0.249 \times (+1)] + [0.286 \times (-1)] + [0.092 \times (-1) \times (+1)] - [0.166 \times (-1) \times (-1)] - [0.271 \times (+1) \times (-1)] =$
 471 0.545; or 3.51 $\mu\text{g g}^{-1}$ on a linear scale.

472

473 Organisms exposed to Cr(III) showed a higher BCF in DG than in G (0.5 vs. 0.15 L g⁻¹), while
474 the opposite was true for Cr(VI) (0.05 vs. 0.22 L g⁻¹). In exposures to the Cr(III) + Cr(VI)
475 mixture, both redox forms showed higher BCFs in G than in DG: 0.08 vs. 0.02 for Cr(III) and
476 0.14 vs. 0.03 for Cr(VI) (all values in L g⁻¹). Calculations of BCF for Cr(III) were done using the
477 geometric means of the measured filterable concentrations at t = 0 and t = 24 h to account for the
478 temporal decrease in Cr concentration (Fig. 1, Table S4). Existing studies on Cr accumulation in
479 bivalves are biased toward Cr(VI) or do not specify Cr speciation in the exposure matrices (Table
480 S6). Potet et al. (2016) exposed two species of dreissenids to Cr(VI) using experimental
481 conditions similar to those adopted in the present study. Despite the differences in the measured
482 Cr burdens, the BCF factors for the two dreissenids (Table S6) are similar to those observed in
483 the present study for *C. fluminea* exposed to Cr(VI). de Oliveira and dos Reis Martinez (2014)
484 exposed specimen of *C. fluminea* along a contamination gradient in a stream impacted by landfill
485 leachate and observed BCF much higher than those reported in the present study (Table S6). We
486 surmise that, during such in situ exposures, the bulk of accumulated Cr originated from particle
487 ingestion rather than uptake of aqueous Cr species. Uptake of particle-bound Cr(III) has been
488 confirmed using radiolabelled material in laboratory studies (Roditi and Fisher, 1999; Wang et
489 al., 1997). Comparison with other published results is less straightforward because of interspecies
490 differences and variability of exposure conditions (Table S6). Nonetheless, our results clearly
491 highlight the need for more research on the bioavailability of Cr(III) or Cr(III) + Cr(VI) mixtures.

492

493 3.3. Biomarker responses

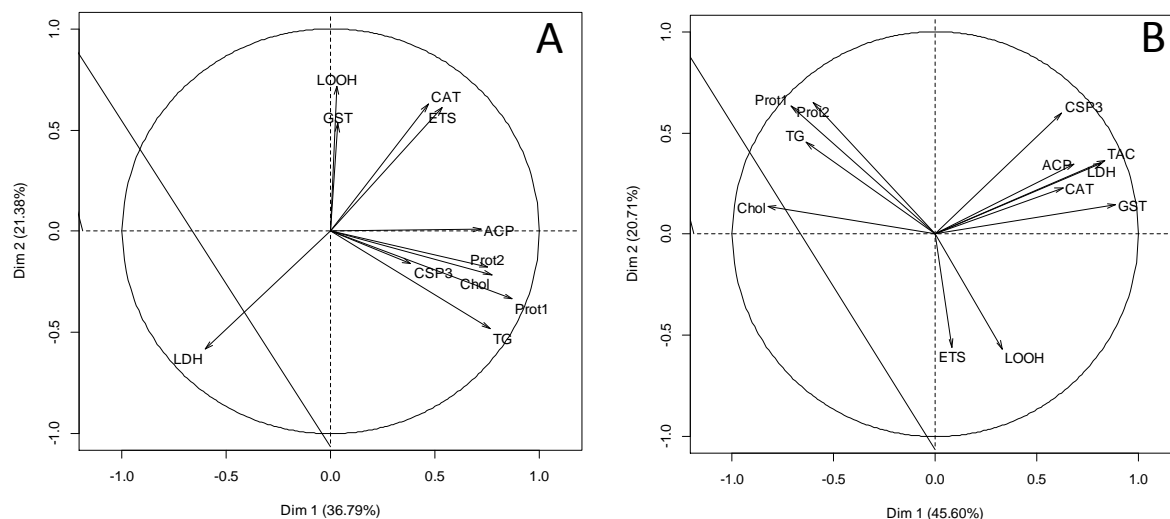
494 Biomarkers expression could be properly measured except for TAC in gills (both experiments)
495 and CAT in gills and digestive glands (exp #2 only). The expression of energy related biomarkers
496 (Prot 1, Prot 2, TG, LDH), Chol and some markers of oxidative stress (TAC, GST and CAT) was

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

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

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

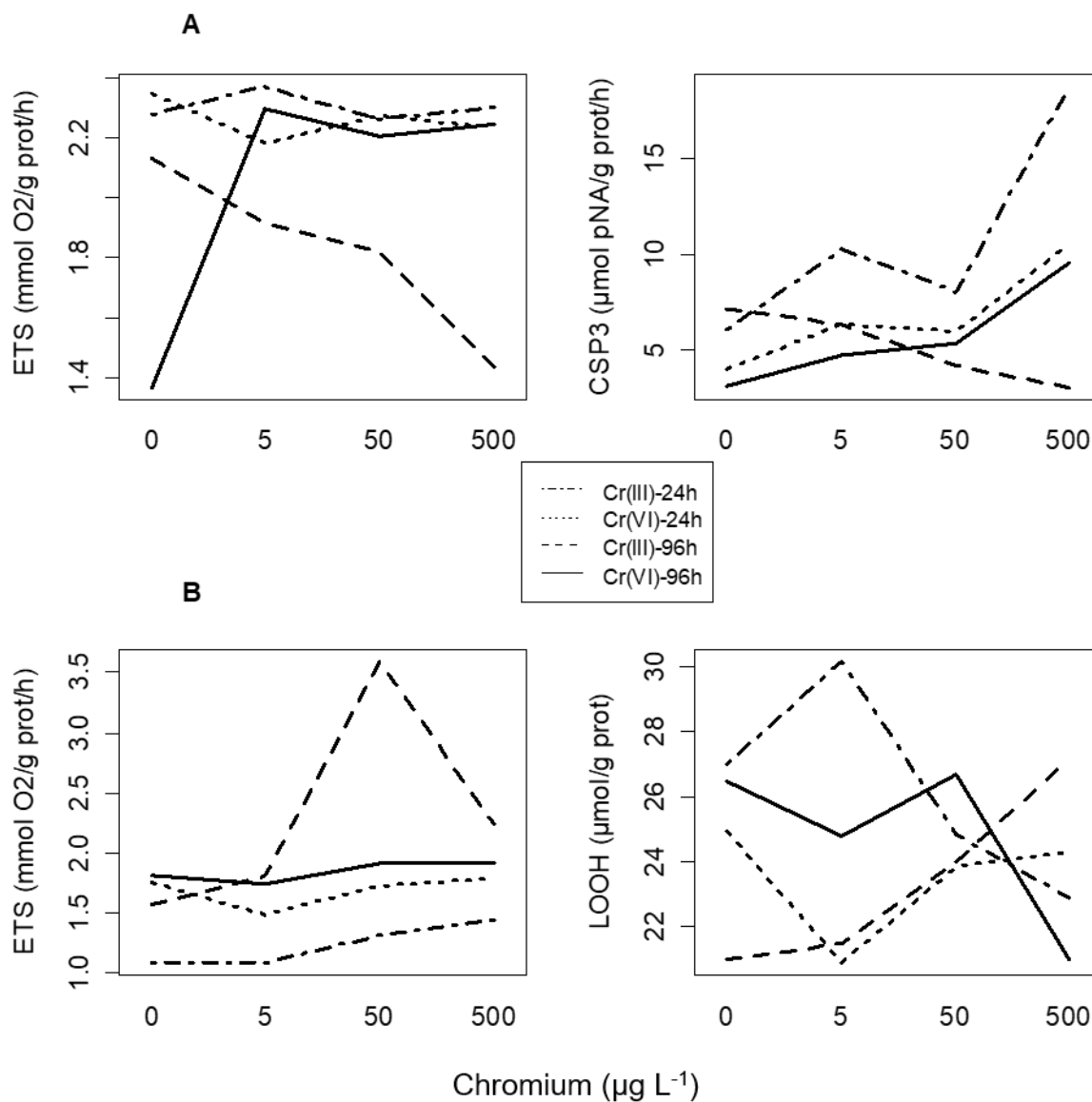
521 Cr redox state and Cr exposure concentration (Table S9). In particular, interaction effects
 522 involving Cr(VI) or Cr(III) concentration were identified for Prot1, LOOH, and CSP3 in DG and
 523 Prot2, ACP, CSP3 and ETS in G (Table S9). In the other cases, interaction effects involved only
 524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



525
 526 Figure 3. Correlation circle graphs of the PCA (A: gills; B: digestive glands) for the biomarker
 527 activities measured in specimen of *Corbicula fluminea* exposed to Cr(III) and Cr(VI) in
 528 experiment 1.

529
 530 Interpreting interaction effects is a complex task and is better illustrated graphically for some
 531 biomarkers with the highest r^2 ; e.g. ETS and CSP3 in G and ETS and LOOH in DG (Table S9
 532 and Fig. 4). The activities of ETS in G at 96 h decreased with increasing Cr(III) concentrations
 533 and increased with increasing Cr(VI) concentrations, but showed no clear trends at 24 h. The
 534 response of CSP3 to Cr(III) showed opposite trends at 24 h and 96 h, while CSP3 levels in
 535 organisms exposed to Cr(VI) increased with concentration regardless of exposure duration. In
 536 DG, ETS levels peaked in specimen exposed to $50\mu\text{g L}^{-1}$ Cr(III) for 96 h, but no clear trends

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



539
 540 Figure 4. Interaction plots for the responses of ETS and CSP3 in gills (A; top graphs) and of ETS
 541 and LOOH in digestive glands (B; bottom graphs) for different combinations of the Cr redox
 542 forms and the exposure time, as a function of Cr concentration in experiment 1.
 543

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

550 For exp #2, neither Cr(III) nor Cr(VI) showed any direct effect on biomarker responses, but
551 differences in biomarker expression existed between tissues. Expression levels of Chol, TG,
552 Prot1, Prot2 and GST were significantly higher ($P < 10^{-4}$) in DG than in G, while the opposite (P
553 < 0.01) was observed for LOOH, CSP3 and ETS (Table S9). The simultaneous exposure to
554 Cr(III) and Cr(VI) did not seem to modify biomarkers responses compared with exposure to
555 individual redox forms of Cr; at least for the selected aqueous exposure levels and the
556 corresponding bioaccumulated concentrations (see section 3.5).

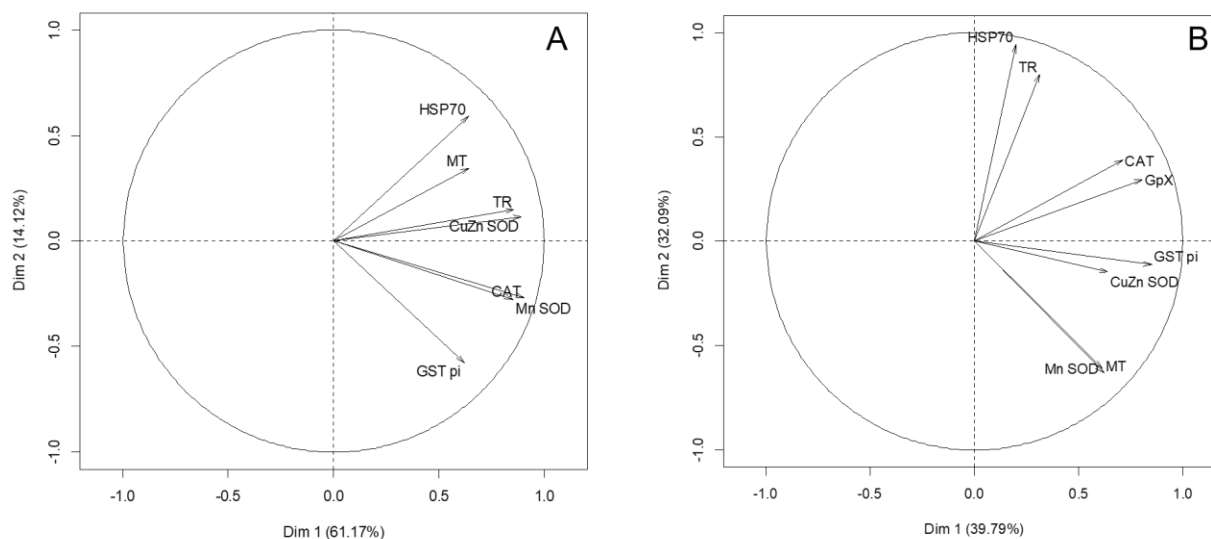
557

558 3.4. Gene expression

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

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

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



571
572 Figure 5. Correlation circle graphs of the PCA (A: gills; B: digestive glands) for the mRNA
573 expression in specimen of *Corbicula fluminea* exposed to Cr(III) and Cr(VI) in experiment 1.

574
575 Changes in the expression levels of GST-pi, CAT, MnSOD, CuZnSOD, MT, TR and HSP70
576 were driven by both Cr redox state and concentration in at least one tissue, while MT expression
577 in digestive glands was driven only by Cr concentration (Table S10). The effects on mRNA
578 expression are mostly marked in the gills compared to the digestive glands (Table S10).
579 Modifications of GST-pi, CAT, MT and HSP70 mRNA levels have been reported in the gills and
580 digestive glands of *Mytilus galloprovincialis* exposed to Cr(VI) (Ciacci et al., 2012; Franzellitti et
581 al., 2012), while we are unaware of previous report for Cr(III) effects on mRNA expression in *C.*
582 *fluminea* or other bivalves. Ciacci et al. (2012) also observed that the effects of Cr(VI) on mRNA

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

585

586

587 **5. Conclusions**

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

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

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

608

609

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615

616

617 **CRedit author statement**

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

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

620 M-N P: Resources, Validation, Writing (Review & Editing)

621 DP: Investigation

622 HP: Investigation

623 JF: Investigation, Methodology, Writing (Review & Editing)

624 FR: Investigation, Methodology, Writing (Original Draft)

625 PR: Investigation

626 MB: Investigation

627 EB: Conceptualization, Supervision, Validation, Funding acquisition, Writing (Original Draft)

628 DAL V: Conceptualization, Supervision, Project Administration, Funding acquisition,
629 Validation, Visualization, Writing (Original Draft)

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Supporting information for

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

812 *Corbicula fluminea*

813

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834 biomarkers measured in gills and digestive glands during exp # 2.

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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.

861

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	GGATCGTTCTCCTGTCAATG	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	GGATCATTGACGAGATGAAGGC	200	200	Response to oxidative stress	1
Ribosomal protein S3	AJ517687	CAGTGTGAGTCCCTGAGATAAAG	AAACGGTTGATAGGACGCAAG	154	250	Translation (Used as housekeeping gene)	5
Thioredoxin reductase	KF275126	AATGCGGTGGCTTCCTACA	TCTGCTGGTCAAATCCTCGC	89	200	Cell redox homeostasis	3

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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.

891 Table S3. Factors, levels and coding values used for regression modelling in the two experiments.
 892 Tissue was not considered as a factor in experiment 1 because data for gills and digestive glands
 893 were treated separately, while only one exposure duration (96 h) was used in experiment 2 (see
 894 text, section 2.2, for details). In the column ‘Factor’, Cr3 and Cr6 are the chosen variable names
 895 for the corresponding Cr redox species.
 896

	Factor	Level	Coding
Experiment 1	Exposure duration (D)	24h	-1
		96h	1
	Chromium redox form (Cr)	Cr3 Cr6	-1 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 $\mu\text{g L}^{-1}$ 50 $\mu\text{g L}^{-1}$	-1 1
Cr6	0 $\mu\text{g L}^{-1}$ 50 $\mu\text{g L}^{-1}$	-1 1	

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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.
 916

	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 \pm 1.29	0.91 \pm 1.33
^{52}Cr control filtered 0.45 μm	0	0.43 \pm 0.43	0.37 \pm 0.49
$^{52}\text{Cr(III)}$ unfiltered – NIA	50	56.4 \pm 2.98	16.1 \pm 3.59
$^{52}\text{Cr(III)}$ filtered 0.45 μm – NIA	50	56.2 \pm 0.93	7.35 \pm 0.81
$^{52}\text{Cr(VI)}$ unfiltered – NIA	50	56.0 \pm 3.89	52.6 \pm 1.81
$^{52}\text{Cr(VI)}$ filtered 0.45 μm – NIA	50	58.5 \pm 1.76	52.7 \pm 2.81
$^{50}\text{Cr(III)}$ unfiltered – IES	100	108 \pm 6.2	26.7 \pm 4.92
$^{50}\text{Cr(III)}$ filtered 0.45 μm – IES	100	104 \pm 1.9	9.89 \pm 1.98
$^{53}\text{Cr(VI)}$ unfiltered – IES	50	45.0 \pm 1.86	42.0 \pm 0.32
$^{53}\text{Cr(VI)}$ filtered 0.45 μm – IES	50	45.0 \pm 0.15	42.0 \pm 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
			n.m.	2.528	8.038
⁵⁰ Cr(III) + ⁵³ Cr(VI) – IES	100 + 50	Gills	n.m.	2.923	7.692
			n.m.	1.891	5.582
			n.m.	2.616	3.911
			n.m.	0.969	1.134
		DG	n.m.	0.232	1.437
			n.m.	1.394	2.002
			n.m.	0.354	0.539
			n.m.		

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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 ($\mu\text{g L}^{-1}$)	Duration (d)	Test medium	Tissue	Cr ($\mu\text{g g}^{-1}$)	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				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
<i>Dreissena r. bugensis</i> ²	4	Cr(VI)	5	4 ^{\$}	Cristaline® mineral water	Whole organism (no shell)	1 ± 0.2	0.2
			15				0.9 ± 0.4	0.06
			5	4 [#]			1.2 ± 0.3	0.24
			15				1.2 ± 0.3	0.08
<i>Mytilus galloprovincialis</i> ³	7	Cr(VI)	0.5	28	Artificial seawater	Whole organism (no shell)	1.4 ± 0.2	2.8
			5				2.5 ± 0.4	0.5
			50				15.4 ± 6.1	0.31

Table S6 (continued)

Organism	Acclimation (d)	Cr form	Cr ($\mu\text{g L}^{-1}$)	Duration (d)	Test medium	Tissue	Cr ($\mu\text{g g}^{-1}$)	BCF (L g^{-1})			
<i>Caelatura companyoi</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			
			144			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	1	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			
				1			4.05 – 9.05	1.1 – 13 ^A			
				0.70 – 3.58			5	DG	3.28 – 14.81	0.9 – 21 ^A	
									15	4.80 – 17.31	1.3 – 25 ^A
									30	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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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 ^S	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 ^S	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
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								
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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	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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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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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																																																																																												
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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991 Table S10. Regression analysis of the mRNA expression (after Log₁₀ transformation) as a
 992 function of the Cr redox form (Cr) and the exposure concentration (Co) after 96 h of exposure
 993 (experiment 1). A probability threshold of 0.01 was used for the construction of all fitted models.
 994 r²: coefficient of determination. G, gills; DG, digestive glands.
 995

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

996 (a) See also table S2 for the coding of Cr redox forms. The coding for Cr(III) is '-1', meaning that mRNA expression
 997 increases with concentration because the minus sign of the coefficient for 'Cr*Co' and the minus sign for Cr(III)
 998 factor give a + sign The opposite situation occurs for Cr(VI).
 999

(b) Insufficient GPx level.

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