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## **Chromium hazard and risk assessment: New insights from a detailed speciation study in a standard test medium.**

Imad Aharchaou, Jean-Sébastien Py, Sébastien Cambier, Jean-Luc Loizeau, Geert Cornelis, Philippe Rousselle, Eric Battaglia, Davide Anselmo Luigi Vignati

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51 **CHROMIUM HAZARD AND RISK ASSESSMENT: NEW INSIGHTS FROM A**  
52 **DETAILED SPECIATION STUDY IN A STANDARD TEST MEDIUM**

53  
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73 **ABSTRACT**

74 Despite the consensus about the importance of chemical speciation in controlling the  
75 bioavailability and ecotoxicity of trace elements, detailed speciation studies during laboratory  
76 ecotoxicity testing remain scarce, contributing to uncertainty when extrapolating laboratory  
77 findings to real field situations in risk assessment. We characterized the speciation and  
78 ecotoxicological effects of Cr<sup>III</sup> and Cr<sup>VI</sup> in the OECD medium for algal ecotoxicity testing.  
79 Total and dissolved (< 0.22 μm) Cr concentrations showed little variability in media spiked  
80 with Cr<sup>VI</sup>, while dissolved Cr concentration decreased by as much as 80% over 72 hours in  
81 medium amended with Cr<sup>III</sup>. Analyses by ion chromatography ICP-MS highlighted the  
82 absence of redox interconversion between Cr<sup>III</sup> or Cr<sup>VI</sup> both in the presence and absence of  
83 algal cells (*Raphidocelis subcapitata*). Furthermore, the concentration of ionic Cr<sup>III</sup> dropped  
84 below detection limits in less than 2 hours with the corresponding formation of carbonate  
85 complexes and Cr hydroxides. Precipitation of Cr<sup>III</sup> in the form of colloidal particles of  
86 variable diameters was confirmed by nanoparticle tracking analysis, spICP-MS and single  
87 particle counting. In terms of time weighted dissolved (< 0.22 μm) Cr concentration, Cr<sup>III</sup> was  
88 4 to 10 times more toxic than Cr<sup>VI</sup>. However, Cr<sup>III</sup> ecotoxicity could arise from interactions  
89 between free ionic Cr<sup>III</sup> and algae at the beginning of the test, from the presence of Cr-bearing  
90 nanoparticles or a combination of the two. Future ecotoxicological studies must pay more  
91 attention to Cr speciation to reliably compare the ecotoxicity of Cr<sup>III</sup> and Cr<sup>VI</sup>.

92  
93 **Keywords**

94 Trace metals, Chromium, Metal speciation, Algae, Hazard/Risk assessment

## 102 INTRODUCTION

103 The bioavailability and ecotoxicity of trace elements in natural environments are determined  
104 by the complex interplay among the ecological traits of living organisms, the toxicodynamics  
105 and toxicokinetics of trace elements, and the elemental speciation in environmental matrices  
106 (Fairbrother et al. 2007; Mason 2013; Harris et al. 2014). Elemental speciation refers to  
107 various physico-chemical forms in which trace elements occur in a given environment and, in  
108 some cases, includes the presence of different oxidation states with contrasting environmental  
109 biogeochemistry. In the specific case of chromium, the importance of speciation is well  
110 appreciated with regard to its biogeochemical cycle (Masscheleyn et al. 1992; Dominik et al.  
111 2007; Saputro et al. 2014) and biological effects (WHO 2009; WHO 2013). In surface waters,  
112 chromium mainly occurs in two oxidation states, namely Cr<sup>VI</sup> and Cr<sup>III</sup>, with contrasting  
113 environmental and biological behavior. Hexavalent chromium, a recognized human  
114 carcinogen, forms negatively charged chemical species, interacts little with colloidal and  
115 particulate material, has a high environmental mobility, and easily crosses biological  
116 membranes (WHO 2013). In contrast, Cr<sup>III</sup> preferentially forms positively charged chemical  
117 species, tends to associate with colloids or suspended particulate matter and is considered of  
118 less ecotoxicological concern (WHO 2009; WHO 2013). In most environmental settings, both  
119 redox forms occur simultaneously, albeit in variable proportions (Bobrowski et al. 2004;  
120 Saputro et al. 2014) and interconversions between them have been documented along with the  
121 corresponding controlling factors (Rai et al. 1989; Richard and Bourg 1991; Lin 2002).  
122 However, experimental research on Cr speciation in standardized ecotoxicological test media  
123 has received limited attention. Thermodynamic speciation calculations are increasingly used  
124 to predict the Cr forms likely to exist in ecotoxicological exposure media, thus aiding  
125 experimental planning and data interpretation (Dazy et al. 2008; Didur et al. 2013; Liu et al.  
126 2015). These calculations also suggest that insoluble species of Cr<sup>III</sup> (mostly Cr oxy-  
127 hydroxides) can form in ecotoxicological test media for algae, daphnids and aquatic plants  
128 (Dazy et al. 2008; Vignati et al. 2010; Ponti et al. 2014). The presence of such chemical  
129 species can modify the fraction of Cr<sup>III</sup> that is potentially bioavailable for the test organisms  
130 during the exposure period, because in most cases it is assumed that the bio-availability of  
131 metals is related to the free ion activity (Morel, 1983). Insoluble species also explain the often  
132 large differences between total and total filterable (< 0.45 µm) Cr concentrations found in test  
133 media spiked with Cr<sup>III</sup>, the extent depending on medium composition (Chapman et al. 1980;  
134 Vignati et al. 2008; Ponti et al. 2014). It should also be noted that filterable (< 0.45 µm) Cr  
135 concentrations do not equal the sum of concentrations of truly dissolved Cr species, because  
136 the initial formation of precipitates may result in solids having particle sizes below this size  
137 cut-off. Furthermore, certain studies present results supporting the conclusion that Cr<sup>III</sup> is  
138 more ecotoxic than Cr<sup>VI</sup> (Holdway 1988; Thompson et al. 2002; Vignati et al. 2010; Lira-  
139 Silva et al. 2011; Kováčik et al. 2015); in contrast with the current consensus considering Cr<sup>VI</sup>  
140 as the most ecotoxic redox form (WHO 2013). A more detailed understanding of Cr  
141 speciation in (standardized) laboratory test media used for ecotoxicity testing will help to both  
142 reconcile contrasting ecotoxicological results in controlled settings and improve our ability to  
143 extrapolate laboratory results to real-field risk assessment situations.

144 On the basis of the above considerations, three aspects related to Cr speciation, and its  
145 changes during a given (standardized) test, appear of particular importance for an improved  
146 understanding of the relative ecotoxicity of Cr<sup>III</sup> and Cr<sup>VI</sup>: a) the stability of (filterable) Cr  
147 concentrations added to the test medium, b) the possibility of redox interconversions between  
148 Cr<sup>III</sup> and Cr<sup>VI</sup>, and c) the formation of Cr oxyhydroxides (i.e., Cr-bearing nanoparticles). In  
149 the present study, the ecotoxicity of three Cr<sup>III</sup> salts and one Cr<sup>VI</sup> salt commonly used in  
150 ecotoxicological studies was investigated using the standard algal medium ISO 8692 (OECD  
151 2011), hereinafter test medium, as exposure matrix and the green alga *Raphidocelis*

152 *subcapitata* as test organism. The behavior and speciation of both Cr<sup>III</sup> and Cr<sup>VI</sup> forms,  
153 including their possible interconversions, were investigated in detail over 72 h; corresponding  
154 to the typical duration of a standard algal test (OECD 2011). Our results showed rapid  
155 changes (i.e., within hours) in Cr<sup>III</sup> speciation which calls for a thorough reconsideration of  
156 the current knowledge about the relative ecotoxicity of Cr<sup>III</sup> and Cr<sup>VI</sup> in terms of both hazard  
157 and risk assessment.

158

## 159 **MATERIALS AND METHODS**

### 160 *Chemicals*

161 All chemicals used were of highest purity. For the various experiments, the test medium  
162 ISO8692 was prepared as described in OECD guideline 201 (OECD 2011) and spiked with  
163 either Cr<sup>VI</sup> as potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, VWR, purity 99.8% min, CAS number 7778-  
164 50-9) or with one of the following Cr<sup>III</sup> salts: chromium chloride (CrCl<sub>3</sub>·6H<sub>2</sub>O; Alfa Aesar,  
165 99.5% min, Crystalline, CAS number 10060-12-5), chromium potassium sulfate  
166 (KCr(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O; VWR, 99%, CAS number 7788-99-0) or chromium nitrate  
167 (Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O; Alfa Aesar, 98.5%, Crystalline, CAS number 7789-02-8).

168 All filtrations were performed using 0.22 μm syringe filters (Millex 33 mm diameter, PVDF,  
169 Millipore, reference number SLGV033NB) prewashed one time with 10 mL of diluted (10%  
170 v/v) hydrochloric acid (HCl Suprapur 30%, Merck Millipore, lot number Z0309518344) and  
171 three times with 10 mL of ultrapure water (Millipore, Q-POD®). A preliminary experiment  
172 showed that Millex syringe filters with nominal pore sizes of 0.22 and 0.45 μm yielded  
173 comparable results for aliquots of test medium amended with 60 μg Cr<sup>III</sup>/L (data not shown).  
174 All samples for the analysis of total Cr concentration were acidified at 1% v/v with  
175 concentrated nitric acid (HNO<sub>3</sub> 67%, Prolabo, VWR, batch number 1108110). A solution of  
176 0.4 M nitric acid (Chemlab, suprapur 70%, batch: 221831609) spiked with 1 μg/L of rhodium  
177 was used as internal standard during the analyses of Cr speciation.

178

### 179 *Chromium ecotoxicity*

180 The ecotoxicity of Cr<sup>VI</sup> and Cr<sup>III</sup> salts to the green alga *Raphidocelis subcapitata* (strain 278/4  
181 originally obtained from the Culture Collection of Algae and Protozoa – CCAP – Argyll,  
182 Scotland, United Kingdom; formerly known as *Pseudokirchneriella subcapitata*) was  
183 evaluated according to OECD guideline 201 (OECD 2011). Tests were performed as  
184 previously described (Vignati et al. 2010) using inoculums of 20,000 cells/mL and nominal  
185 exposure concentrations of 9, 18, 35, 70, 140, 280, 560, 1125, 2250 μg Cr/L. The effects of Cr  
186 salts on algal growth were expressed as the effective exposure concentrations (ECs<sub>50</sub>) that  
187 produced a 50% decrease in algal fluorescence (BMG LabTechnologies, Fluostar), considered  
188 as a proxy for algal biomass. The ECs<sub>50</sub> were calculated by performing a nonlinear regression  
189 on Hill's model with the macro REGTOX (Version EV7.0.4., E. Vindimian) (Vindimian  
190 2010). In the case of Cr<sup>III</sup>, ECs<sub>50</sub> were estimated based on both nominal concentrations and  
191 Time-Weighted Mean (TWM) dissolved measured concentrations (OECD 2012). Starting  
192 from this point, the terms total and dissolved concentrations will indicate the unfiltered and  
193 filtered (0.22 μm) portions of measured Cr levels.

194

### 195 *Temporal evolution of Cr concentration in the test medium*

196 Aliquots (50 mL) of freshly prepared test medium were transferred to acid-washed  
197 Erlenmeyer flasks (150 mL) and spiked with a Cr concentration corresponding to the ECs<sub>50</sub>  
198 of either Cr<sup>III</sup> (added as CrCl<sub>3</sub>·6H<sub>2</sub>O) or Cr<sup>VI</sup> (added as K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) in the presence or absence of  
199 an initial algal inoculum of 20,000 cells/mL. Aliquots were analyzed for total and dissolved  
200 Cr after 0.5, 1, 2, 4, 8, 24, 48, and 72 h of incubation under continuous light (90 – 110  
201 μE/m<sup>2</sup>/s) at 21 ± 2 °C and, for biotic samples only, rotary agitation (100 rpm). For dissolved

202 Cr determination, the first 5 mL of filtered solution were used for preconditioning the filters  
203 and discarded. Preconditioning aimed at removing possible residues of ultrapure water from  
204 filter pores and saturate possible sorption sites for Cr. Total and dissolved Cr concentrations  
205 were determined by flame atomic absorption spectrometry (FAAS, Perkin-Elmer AAnalyst  
206 100) or, for concentrations < 20 µg/L, graphite furnace atomic absorption spectrometry  
207 (GFAAS, Varian spectra 300, using 0.1% Mg(NO<sub>3</sub>)<sub>2</sub> as matrix modifier according to  
208 manufacturer instructions). Quantification limits were 10 µg/L for FAAS and 0.2 µg/L for  
209 GFAAS. Certified reference materials SPS-SW1 (2.00 ± 0.02 µg/L) and WW1 (200 ± 1  
210 µg/L), both from SpectraPure standards (Manglerud, Norway), were used as quality controls.  
211 All analytical results are expressed in µg Cr/L.

212 The statistical significance of the temporal changes in Cr concentrations was evaluated by  
213 one-way ANOVA with Tukey post-hoc comparison. Visual Minteq modelling (ver 3.1) was  
214 done to investigate Cr<sup>III</sup> speciation in test medium amended with an initial total concentration  
215 of 60 µg Cr/L. No redox transformations were assumed, because it will be argued based on  
216 experimental proof that these did not occur. Calculations were performed using  
217 experimentally measured Cr concentrations and pH values, and the test medium composition  
218 as reported in OECD (2011). No modifications were made to the Visual Minteq default  
219 database and the Debye-Hückel equation was used for activity corrections.

#### 220 221 *Chromium redox speciation*

222 To investigate Cr redox speciation and the possible interconversion between Cr<sup>III</sup> and Cr<sup>VI</sup>,  
223 aliquots (50 mL) of the test medium were spiked with either Cr<sup>VI</sup> or Cr<sup>III</sup> (added as chloride)  
224 both in absence (abiotic medium) and presence (biotic medium) of algal inoculums (20,000  
225 cells/mL). Nominal Cr concentrations in the spiked solutions were 60 µg/L for Cr<sup>III</sup> and 115  
226 µg/L for Cr<sup>VI</sup>, corresponding to the calculated 72 h ECs50 (see section Chromium ecotoxicity  
227 in Results and discussion). Samples were analyzed immediately after spiking (t=0) or  
228 incubated at 23 ± 1 °C, under continuous light (90 – 110 µE/m<sup>2</sup>/s) and, for biotic samples  
229 only, rotary agitation (100 rpm) for 24, 48 and 72 h prior to analysis (Figure S1). At the end  
230 of each incubation period, sample aliquots for Cr speciation were filtered as described above.  
231 After preconditioning the filters with 5 mL (subsequently discarded) of Cr-amended test  
232 medium, 10 mL of filtrate was collected in polystyrene sample vials and immediately  
233 analyzed by ion chromatography-inductively coupled plasma mass spectrometry (IC-ICP-  
234 MS). Samples were kept at between 4 and 6 °C in a refrigerated sample tray during analysis.  
235 An additional abiotic sample of test medium (2 L) was prepared in an acid-washed low-  
236 density polyethylene bottle and spiked with 60 µg/L of Cr<sup>III</sup> to follow more closely the  
237 kinetics of the changes in Cr<sup>III</sup> speciation (Sacher et al. 1999; Séby et al. 2003). Aliquots for  
238 analysis were recovered every 20 min over the first 6 hours after spiking and again after 24,  
239 48, and 72 hours of incubation in the same conditions described above.

240 Trivalent Cr and hexavalent Cr species were separated and quantified by IC-ICP-MS using a  
241 Thermo ICS 5000+ pump coupled with an anion exchange column (Thermo AG7 2x50 mm)  
242 and connected to the nebulizer of an ICP-MS (Thermo, Series XII) (Table S1). A solution of  
243 0.4 M nitric acid spiked with 1 µg/L of rhodium was used to elute the Cr species from the  
244 anion exchange column (see Table S1 for more details). The analyses were carried out  
245 according to the method developed by the French Agency for Food, Environmental &  
246 Occupational Health & Safety (ANSES) on the basis of the application note from the supplier  
247 (Sacher et al. 1999; ANSES/LHN/MT-CrVI). Chromium detection was performed at m/z 52  
248 and 53. Experimental IC-ICP-MS results were compared with those of speciation modeling  
249 carried out as described previously (section Temporal evolution of Cr concentration) using  
250 Visual Minteq.

251

252 *Formation of Cr nanoparticles in the test medium*

253 Aliquots of test medium were spiked with 60 µg/L of Cr<sup>III</sup> (added as chloride or nitrate) and  
254 the formation of Cr-containing particles was investigated by Nanoparticle Tracking Analysis  
255 (NTA), spICP-MS (single particle ICP-MS) and Single Particle Counting (SPC) over 72 h;  
256 that is the typical duration of an algal ecotoxicological test. Blank samples consisting of  
257 unspiked test medium were systematically analyzed along with the corresponding samples.  
258 Total and dissolved Cr concentrations were determined by FAAS during the various  
259 experiments.

260 For NTA (Nanosight NS500; Malvern instruments Ltd, UK), spiked samples were aged for 0,  
261 0.5, 1, 2, 24, 48, and 72 h and assayed (before and after filtration) for particle concentration  
262 and particle size distribution (PSD). The NS500 provides detailed analysis of the  
263 concentration and size distribution of all types of nanoparticles from 10 nm to 1000 nm in  
264 diameter (Carr and Wright 2013) using the properties of both light scattering and Brownian  
265 motion in liquid suspensions. Particles in suspension in the sample chamber (volume = 0.3  
266 mL) were visualized using a 20x magnification microscope equipped with a camera operated  
267 at 25 frames per second for 60 s. The resulting video files were elaborated using the built-in  
268 software (NTA 2.3 build 0033) to calculate the hydrodynamic diameter of the individually  
269 tracked particles. After each measurement, the sample chamber was flushed with milliQ water  
270 and reloaded with the next sample aliquot. All measurements were done in triplicate and the  
271 mean ( $\pm$  one standard deviation) of particle concentration and average size calculated (see  
272 supplementary information for protocol optimization).

273 While NTA determines the total particle count, spICP-MS (Perkin Elmer NexION 350D ICP-  
274 MS) was used to specifically measure Cr-containing particles. Samples were aged for 0, 24,  
275 48 and 72 h and diluted to a theoretical concentration of 0.06 µg/L total Cr before analysis.  
276 The transport efficiency was calculated based on a dissolved standard curve of Au stabilized  
277 by 0.1 % cysteine and on the TEM-based median size (56 nm) of gold nanoparticles  
278 (NIST8013, nominal diameter 60 nm) using the method of Pace et al. (2011). Data acquisition  
279 was performed in fast acquisition scanning technique (FAST) mode with a dwell time of 50  
280 µs over 100 s. Raw data (two million readings per sample) were extracted and loaded into the  
281 Nanocount software (<http://blogg.slu.se/nanocount/>) for the determination of particle number  
282 and particle size distribution. Correction for dissolved concentrations was performed using  
283 outlier analysis based on a  $5 \times \sigma$  criterion specifically developed for FAST data (Tuoriniemi  
284 et al. 2015). Peak integration time was set to 20 ms and the minimum detectable cluster was  
285 set at 4 ions. Distributions of the corresponding spherical size were calculated assuming a  
286 Cr(OH)<sub>3</sub> ( $\rho = 3.11 \text{ g/cm}^3$ ) composition and a spherical shape. It should be noted that these  
287 were assumptions as the exact composition and shape of the formed particles are unknown.

288 However, considering the measured pH range of the test medium initially spiked with 60 µg  
289 Cr/L (7.8–7.9 units), trivalent chromium is expected to rapidly undergo hydrolysis and form  
290 amorphous oxyhydroxides (Rai et al. 1989; Kotaš and Stasicka 2000). The assumption on  
291 particle shape would allow comparison with the results obtained using other methods.  
292 To detect the presence of particles outside the NTA and spICP-MS measuring range, samples  
293 were analyzed after 0, 24, 48, and 72 hours of incubation using three Single Particle Counters  
294 (SPCs): a high sensitivity in-situ monitor (HSLIS) Model M50 for the range 50 to 200 nm and  
295 two Volumetric Spectrometers (LiQuilaz-S02 and LiQuilaz-S05, Particle Measuring Systems,  
296 Boulder, CO, U.S.A) for the ranges 200 to 2000 nm and 500 nm to 20 µm, respectively. The  
297 SPCs use light scattering to count individual particles across 31 size classes with some  
298 overlapping between the LiQuilaz-S02 and S05 (Rossé and Loizeau 2003). Results for spiked  
299 medium were corrected for blank values.

300  
301

## 302 RESULTS AND DISCUSSION

### 303 *Chromium ecotoxicity and temporal evolution of Cr concentrations*

304 Results of ecotoxicity tests were highly reproducible with ECs50 at 72 h (mean value  $\pm$  one  
305 standard deviation) of  $113 \pm 3$   $\mu\text{g/L}$  ( $n = 3$ ) for  $\text{K}_2\text{Cr}_2\text{O}_7$ ,  $59 \pm 3$   $\mu\text{g/L}$  for  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$  ( $n = 6$ ),  
306  $61 \pm 8$   $\mu\text{g/L}$  for  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  ( $n = 3$ ) and  $62 \pm 3$   $\mu\text{g/L}$  for  $\text{KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  ( $n=3$ ). The  
307 ECs50 values reported in the literature for freshwater algae range from 130 to 470  $\mu\text{g/L}$  for  
308  $\text{Cr}^{\text{VI}}$  and from 30  $\mu\text{g/L}$  to over 1000  $\mu\text{g/L}$  for  $\text{Cr}^{\text{III}}$  (WHO 2009; Vignati et al. 2010; OECD  
309 2011; WHO 2013). In the case of  $\text{Cr}^{\text{VI}}$ , a ring test involving 16 laboratories determined a  
310 range of 72h-ECs50 between 70 and 260  $\mu\text{g Cr/L}$  for *Scenedesmus subspicatus* and  
311 *Selenastrum capricornutum* (a former name for *R. subcapitata*) based on algal biomass  
312 endpoint (Munn et al. 2005; WHO 2013); which agrees with our results. In the case of  $\text{Cr}^{\text{III}}$ ,  
313 our results are in the low range of the values reported in the literature and indicate a higher  
314 toxicity of  $\text{Cr}^{\text{III}}$  compared with  $\text{Cr}^{\text{VI}}$ ; an occurrence also reported in previous research  
315 (Holdway 1988; Thompson et al. 2002; Vignati et al. 2010; Lira-Silva et al. 2011; Kováčik et  
316 al. 2015). Further insights in the relative ecotoxicity of the two redox forms of Cr can be  
317 gained by examining their behavior in the test medium. In particular, the detailed examination  
318 of  $\text{Cr}^{\text{VI}}$  and  $\text{Cr}^{\text{III}}$  behavior added to the test medium at concentrations corresponding to their  
319 respective ECs50 allows to exactly determine the actual concentrations responsible for the  
320 observed effects.

321 At  $t = 0$  h, the measured total and dissolved Cr concentrations (added as  $\text{Cr}^{\text{III}}$ ) were similar  
322 and corresponded to the expected nominal values in both abiotic and biotic media. In the  
323 absence of algae, total Cr concentrations remained within 20% of the initial value over the  
324 first 24 hours and had decreased by about 40% after 72 h. In the same experiment, dissolved  
325 Cr concentrations remained within 20% of the initial values only over the first two hours, but  
326 had dropped by 40% eight hours after spiking and by 83% after 72 h (Figure 1). The decrease  
327 in total Cr concentrations indicates that some of the added Cr may be lost to the walls of the  
328 test vessel over time. However, comparison of total and dissolved concentrations indicates  
329 that ca. 50% of the added Cr likely occurs in the form of colloidal or particulate form (most  
330 likely amorphous Cr oxyhydroxides) starting from 24 hours after spiking (i.e., for over 2/3 of  
331 the test duration). Calculations in Visual Minteq for an initial concentration of 60  $\mu\text{g/L}$  as  $\text{Cr}^{\text{III}}$   
332 predicted that, in the observed pH range of 7.8 to 7.9, the  $\text{Cr}(\text{OH})_3$  species should account for  
333 92–94 % of the total Cr. Furthermore, about 80% of the initially added  $\text{Cr}^{\text{III}}$  was predicted to  
334 precipitate in the form of amorphous  $\text{Cr}(\text{OH})_3$ , in very good agreement with the 80% decrease  
335 of the corresponding dissolved Cr concentration over 72h (Figure 1). Colloid/nanoparticle  
336 formation was indeed confirmed using NTA and spICP-MS (see section Formation of Cr  
337 nanoparticles in Results and Discussion). Analogous calculations using the dissolved Cr  
338 concentration measured at 72h ( $11 \pm 0.25$   $\mu\text{g/Cr L}$ ,  $n=3$ ) also predicted that  $\text{Cr}(\text{OH})_3$  would  
339 account for over 90% of the measured Cr concentration, but with less than 2% occurring in  
340 the precipitated phase. In both cases, the sum of the positively charged species  $\text{Cr}(\text{OH})_2^+$  and  
341  $\text{CrOH}^{2+}$  was predicted to represent about 3.5% of the total dissolved Cr and EDTA complexes  
342 were about 2.5%. Further comparison between speciation calculations and analytical results is  
343 presented in the next section.

344 The difference between total and dissolved Cr concentrations becomes statistically significant  
345 starting from 8 h after spiking (one-way Anova with Tukey post-hoc comparison,  $p < 0.01$ ).  
346 Considering the temporal decrease of the dissolved Cr concentration, assumed as a better  
347 proxy of the Cr potentially available to the algae (see de Paiva Magalhães et al. 2015 and  
348 references therein for a detailed discussion), the time-weighted mean (TMW) (OECD 2012)  
349 exposure concentration of the algae to Cr (added as  $\text{Cr}^{\text{III}}$ ) was about 25  $\mu\text{g/L}$  for an initial  
350 addition of 60  $\mu\text{g/L}$  as  $\text{Cr}^{\text{III}}$ . Similar behavior of total and dissolved Cr concentration was  
351 observed over the concentration range 20 – 7,000  $\mu\text{g/L}$  for  $\text{CrCl}_3$  and for selected



352 concentrations of  $\text{Cr}(\text{NO}_3)_3$  and  $\text{CrKSO}_4$  (Figures S2–S4); confirming that the decrease in the  
353 dissolved exposure concentrations must be considered and quantified when testing  $\text{Cr}^{\text{III}}$   
354 toxicity in standardized laboratory media. These observations corroborate previous findings  
355 obtained by measuring the decrease in Cr concentrations in test medium directly in 96-well  
356 microplates in the absence of algae and without actually providing information on the total vs.  
357 dissolved Cr concentrations (Vignati et al. 2008; Vignati et al. 2010).  
358 In the presence of algae, both total and dissolved Cr concentrations decreased with time  
359 (Figure 1) and the decrease was more marked for the dissolved concentrations (over 95% after  
360 72 hours). The incomplete recovery of total Cr was likely caused by attachment of algal cells  
361 to the vessel's walls despite the constant agitation. Most importantly, the differences between  
362 dissolved Cr concentrations in the absence and presence of algae became statistically  
363 significant starting from 24 h after spiking (one-way Anova with Tukey post-hoc comparison,  
364  $p < 0.01$ ), indicating adsorption onto or absorption into the algal cells of most of the available  
365 dissolved Cr. Based on the dissolved Cr concentrations in the presence of algae, the 72 h  
366 TMW effect concentration (EC50) for algal growth (in terms of biomass) was  $11.5 \mu\text{g/L}$ .  
367 Analogous experiments performed with aliquots of test medium amended with  $\text{Cr}^{\text{VI}}$  at the  
368 corresponding EC50 concentration ( $115 \mu\text{g/L}$ ) did not show significant changes in total or  
369 dissolved Cr concentration neither in the absence nor in the presence of algae (Figure S5).  
370 The above observations lead us to the conclusion that  $\text{Cr}^{\text{III}}$  is 4 to 10 times more toxic than  
371  $\text{Cr}^{\text{VI}}$  when the 72 h EC50 for  $\text{Cr}^{\text{III}}$  is expressed as the TWM dissolved concentration estimated  
372 from measurements in the absence or presence of algae, respectively. However, the presence  
373 of algae had no measurable effect on dissolved  $\text{Cr}^{\text{VI}}$  concentrations (Figure S6). One  
374 possibility is that the entrance of minimal quantities of  $\text{Cr}^{\text{VI}}$  into the cells may suffice to  
375 reduce algal biomass, which would support the current consensus that  $\text{Cr}^{\text{VI}}$  is more toxic than  
376  $\text{Cr}^{\text{III}}$ . On the other hand, exposure to  $\text{Cr}^{\text{VI}}$  has been shown to cause lysis of *R. subcapitata*  
377 cells at concentrations of  $1.75 \text{ mg/L}$  or higher added to an algal inoculum of  $2 \times 10^5 \text{ cells/mL}$   
378 (Labra et al. 2007); which corresponds to an initial quantity of  $8.75 \text{ ng}$  of  $\text{Cr}^{\text{VI}}$  per algal cell at  
379 the beginning of the experiment (our calculation). This figure compares favorably with the  
380 value of  $5.75 \text{ ng}$  of  $\text{Cr}^{\text{VI}}$  per algal cell in our experimental conditions for an initial spike of  
381  $115 \mu\text{g/L}$ . Upon cell lysis, the initially internalized  $\text{Cr}^{\text{VI}}$  would be released back to the test  
382 medium resulting in an apparent stable total (dissolved) Cr concentration over the duration of  
383 the experiment. Note that  $\text{Cr}^{\text{VI}}$  can undergo intracellular reduction to  $\text{Cr}^{\text{III}}$  (Cheung and Gu  
384 2007; Volland et al. 2012; Kováčik et al. 2015) which could also be released back to the test  
385 medium upon cell lysis; possibly in association with intracellular constituents preventing its  
386 precipitation in the test medium. While no experiments were performed to determine if cell  
387 lysis had occurred, we verified possible changes in the redox state of Cr in the test medium  
388 over the exposure duration.

#### 389 *Chromium redox speciation*

391 Aliquots of test medium spiked with  $\text{Cr}^{\text{VI}}$  exhibited a single peak, corresponding to the one  
392 observed in a standard solution of  $\text{Cr}^{\text{VI}}$ , with a retention time of about 50 s regardless of the  
393 presence of algae and of the incubation time (Figures 2A and S6). The total  $\text{Cr}^{\text{VI}}$   
394 concentration, determined from total peak area, was  $118 \mu\text{g/L}$  at  $t=0$  and  $120 \mu\text{g/L}$  after 72h  
395 of incubation in the abiotic test medium and  $137 \mu\text{g/L}$  at  $t=0$  and  $120 \mu\text{g/L}$  after 72h in the  
396 biotic medium. No peaks corresponding to  $\text{Cr}^{\text{III}}$  were observed in either abiotic or biotic test  
397 medium (Figures 2A and S6). While this result does not provide information on possible  $\text{Cr}^{\text{VI}}$   
398 to  $\text{Cr}^{\text{III}}$  interconversion in the intracellular environment (Shanker et al. 2005; Viti et al. 2014;  
399 Kováčik et al. 2015), it shows that algal cells are exposed to a practically constant  
400 concentration of  $\text{Cr}^{\text{VI}}$  throughout the test duration. The corresponding ECs50 values for  $\text{Cr}^{\text{VI}}$

401 can therefore be confidently ascribed to this specific redox form and calculated from the  
402 concentrations initially added to the test medium.

403 On the other hand, marked and rapid changes in Cr speciation occurred in medium aliquots  
404 amended with Cr<sup>III</sup>. At t=0 and in the absence of algae, a single peak at a retention time of  
405 about 100 s, corresponding to the hydrated Cr<sup>III</sup> ion, was observed (Figure 2B). The  
406 corresponding dissolved Cr<sup>III</sup> concentration determined by IC-ICP-MS was 57 µg/L, in  
407 agreement with the expected value of 60 µg/L. However, after 24 h of incubation, the peak  
408 area at 100 s had dropped by 99% and the corresponding dissolved Cr<sup>III</sup> concentration at 72 h  
409 was only 0.39 µg/L (Figure 2B). Starting from 24 h after spiking, two additional peaks were  
410 observed at retention times around 45 s and 75 s (Figure 2B). Considering the measured pH  
411 range of the test medium (7.5–7.9 for medium aliquots amended with Cr<sup>III</sup> concentrations  
412 between 20 and 1250 µg Cr/L), Cr<sup>III</sup> is expected to rapidly undergo hydrolysis (Rai et al.  
413 1989; Richard and Bourg 1991; Kotaš and Stasicka 2000) and the peaks at 45 and 75 s likely  
414 correspond to the hydrolytic Cr<sup>III</sup> complexes Cr(OH)<sup>2+</sup> and Cr(OH)<sub>3</sub><sup>0</sup> (Séby et al.  
415 2003). Addition of 5 µg/L of Cr<sup>VI</sup> to an aliquot of the solution initially spiked with 60 µg/L of  
416 Cr<sup>III</sup> and aged for 4 h resulted in the expected Cr<sup>VI</sup> peak at 50 s, thus confirming that the peak  
417 at 45 s was not the results of an oxidation of Cr<sup>III</sup> to Cr<sup>VI</sup> during the experiment (Figures 2C  
418 and S6).

419 As already seen in the previous section, for an initial concentration of 60 µg/L of Cr<sup>III</sup>,  
420 speciation calculations using Visual Minteq suggest that, at equilibrium, the positively  
421 charged species Cr(OH)<sub>2</sub><sup>+</sup> and CrOH<sup>2+</sup> should represent about 3.5% of the total in the  
422 observed pH range of 7.8–7.9. Assuming a dissolved Cr concentration of 11 µg Cr/L after 72h  
423 of incubation (see previous section and figure 1), the theoretical concentration of positively  
424 charged Cr species at the end of the exposure period would be 0.2–0.4 µg/L; in agreement  
425 with the IC-ICP-MS speciation results (Figure 2B).

426 Samples spiked with Cr<sup>III</sup> in the presence of algae showed the three distinct peaks at retention  
427 times of about 100 s, 75 s, and 45 s already at t = 0 (Figure S6). The area of all peaks rapidly  
428 decreased and became practically negligible after 24 hours of incubation (Figure S6). In the  
429 presence of algae, the concentration of hydrated Cr<sup>III</sup> (peak at 100 s) decreased from 30 µg/L  
430 at t = 0 to 0.14 µg/L at t = 72 h (Figure S6). The discrepancy between the measured (30 µg/L)  
431 and expected concentration (60 µg/L) at t = 0 can be explained by a more rapid formation of  
432 hydrolysis products. Indeed, the presence of particular microenvironments with increased pH  
433 around algal cells and colonies was reported to enhance Fe hydrolysis (Sunda and Huntsman  
434 2003) and an analogous phenomenon may occur in the case of Cr<sup>III</sup>. Again, no peak indicative  
435 of the formation of Cr<sup>VI</sup> was identified throughout the experiment duration so that the  
436 ecotoxicological effects discussed previously (section Chromium ecotoxicity in Results and  
437 Discussion) can be ascribed to Cr<sup>III</sup>.

438 The disappearance of the chromatographic peak of hydrated Cr<sup>III</sup> (t = 100 s) between 0 and 24  
439 h of incubation prompted us to examine the changes in Cr<sup>III</sup> speciation at shorter time  
440 intervals. Note that, given the absence of calibration standards for the peaks at 45 and 75 s,  
441 the changes in the relative importance of the chromatographic peaks can be discussed solely  
442 on the basis of their signal intensities. Immediately after spiking, 84% of dissolved Cr was  
443 present as hydrated Cr<sup>III</sup> (retention time 100 s) with the peaks at 75 s and 45 s representing  
444 about 8% of the total signal each (Figures 2C and 3). The peak of hydrated ionic Cr<sup>III</sup> at 100 s  
445 decreased very rapidly and was only 2% of the initial dissolved Cr concentration after 6 hours  
446 of incubation (Figure 3). At the same time, the peak at 75s increased up to 56% of the total  
447 signal 1 hour after spiking and then slowly decreased to 40% after 6 h. The peak at 45 s  
448 accounted for 7 and 10% of the total signal regardless of the incubation time (Figure 3). Both  
449 the sum of the signals of the three peaks and the corresponding dissolved Cr signal (assayed

450 by ICP-MS) decreased by 50% and 40% compared with their respective initial values (Figure  
451 3).

452 Samples collected after 24, 48, and 72 h of incubation confirmed the initial decreasing trend  
453 and, in the case of dissolved Cr, were comparable with the results obtained by FAAS (Figure  
454 1). The difference between dissolved Cr (ICP-MS) and the sum of various Cr peaks (IC-ICP-  
455 MS) may be due to nanoparticle formation depositing in the column and going undetected. On  
456 the other hand, the signal of total Cr (in terms of relative intensity measured by ICP-MS)  
457 remained within 20% of the initial value (Figure 3). Overall, the rapid changes in Cr<sup>III</sup>  
458 speciation added to the test medium (particularly the disappearance of ionic Cr<sup>III</sup> in less than 2  
459 h) arise the question of which Cr<sup>III</sup> species are actually responsible for the observed decrease  
460 in algal biomass during ecotoxicity testing. These considerations will be developed later in the  
461 text (section Implications for Cr hazard and risk assessment) after the presentation of the  
462 results confirming the formation of Cr-bearing particles in the test medium.

463

#### 464 *Formation of chromium (nano)particles*

465 NTA analysis showed that colloidal particles formed within minutes after adding soluble Cr<sup>III</sup>  
466 into the test medium. Particle concentration and size ranged between  $9.64 \times 10^8$  and  $29.7 \times$   
467  $10^8$  particles/mL and 80 and 140 nm, respectively (Figure 4). The particle concentration was  
468  $0.17 \pm 0.10 \times 10^8$  particles/mL in test medium without added Cr<sup>III</sup> (Figure 4). Filtration (0.22  
469  $\mu\text{m}$ ) of Cr<sup>III</sup>-amended aliquots of test medium lowered particle concentration to around  $0.6 \times$   
470  $10^8$  particles/mL, but did not decrease their size by more than 25% (Figure 4). Total Cr  
471 concentration remained within  $\pm 20\%$  of initial value over the entire experiment duration.  
472 Dissolved Cr concentrations also did not change during the first 2 h of incubation, but then  
473 progressively decreased to 26% of the initial value after 72h (Figure 4). The concomitant  
474 removal of particles and decrease in dissolved Cr concentration (Figure 4) confirms the  
475 formation of Cr containing (nano)particles starting from initially soluble Cr<sup>III</sup> salts for  
476 incubation times  $> 24$  h (Figure 4). The experimental decrease of dissolved Cr concentrations  
477 is in agreement with the theoretical calculation performed with Visual Minteq (see Chromium  
478 ecotoxicity in Results and discussion).

479 Analysis by spICP-MS confirmed that the nanoparticles formed immediately after spiking  
480 actually contained Cr (Figure S11). The orders of magnitude of size is similar to those  
481 measured using NTA (Figure S11 and Figure 4), at least when using the assumed composition  
482 and shape (Table S3). On the other hand, spICP-MS detected an increase in particle number  
483 from  $5 \times 10^6$  ( $t \leq 24$  h) to  $2 \times 10^8$  ( $t \geq 72$  h), suggesting that the mass percentage of Cr<sup>III</sup>  
484 occurring as particles increased from 4% at 0 h to 20% at 72 h to 76% at 96 h. Differences in  
485 particle numbers estimated by spICP-MS and NTA likely arise from the specific technical  
486 features of the two methods. Large particles do not manage to pass a spray chamber and are  
487 therefore not measured during spICP-MS (Tuoriniemi et al. 2017), which therefore tends to  
488 favor small particles as long as they are larger than the detection limit of approx. 90 nm.

489 NTA, in contrast, preferentially detects relatively large particles that scatter more light and is  
490 less accurate in detecting relatively small particles. Note also that the particle size  
491 distributions detected with spICP-MS abruptly stop at the size detection limit. Thus, despite  
492 the two techniques yielding similar average particle sizes, NTA likely observes a fairly  
493 constant number concentration of relatively large particles that are mostly removed by  
494 filtration, while spICPMS observes an increase in the number of (relatively small) particles  
495 with time (Figure 4 and S11). At short incubation times, a very large mass of Cr may reside in  
496 the particle fraction that can pass a 0.22  $\mu\text{m}$  filter without being detected by neither NTA nor  
497 spICP-MS. Hence, a decrease Cr mass concentration is not observed when filtering samples at  
498 0.22  $\mu\text{m}$  at short incubation times (Figure 4). Measurement of total and dissolved Fe (data not  
499 shown) allowed to exclude the formation of Fe-bearing particles (including mixed Cr-

500 Fe(oxy)hydroxides) (Sass and Rai 1987) and thus the possibility of indirect ecotoxic effect of  
501 Cr<sup>III</sup> via sequestration of the essential nutrient Fe. Interestingly, model calculations with  
502 Visual Minteq predicted that the test medium should be oversaturated with respect to different  
503 mineral phases including several Fe-containing minerals and hydroxyapatite. The exact  
504 chemical nature of particles formed in Cr-spiked test medium clearly deserves further  
505 experimental investigation, especially at short incubation times for which the assumption of  
506 equilibrium is unlikely to be valid. Finally, removal of particles with sizes between 80 and  
507 140 nm by filters with a 0.22 µm nominal cut-off can be explained by an actual smaller cut-  
508 off of the filters' pores (She et al. 2008) or by the formation of larger particles undetectable  
509 via NTA and spICP-MS.

510 Measurements by SPC confirmed the presence of particles in the same range identified by  
511 NTA and spICP-MS, but also of particles larger than 300 nm in unfiltered test medium spiked  
512 with Cr<sup>III</sup> and incubated for 24, 48, and 72 hours (Figure 5 and S7). At t=0, only particles  
513 between 50 and 150 nm were observed at a concentration of  $3.2 \times 10^5$  particles/mL. After 24  
514 hours, the concentration of particles between 50 and 200 nm increased to  $6.1 \times 10^6$   
515 particles/mL and particles larger than 300 nm ( $8.5 \times 10^3$  particles/mL) had also appeared.  
516 Particles in the range 50–200 nm were still present after 48 ( $6.5 \times 10^6$  particles/mL) and 72 h  
517 ( $9.8 \times 10^6$  particles/mL) of incubation, while particles larger than 300 nm eventually  
518 disappeared after 72 h (Figure 5). The latter observation suggests the formation of particles  
519 too large to be measured by the instruments or, more likely, settling too quickly to be  
520 effectively sampled by the instrument.

521 Based on the analysis of total and dissolved Cr in the samples subject to SPC measurements  
522 (figure S7), 45 µg of Cr (75% of the Cr<sup>III</sup> initially added to the test medium) had been  
523 incorporated in the observed particles after 72 hours. This result agrees well with the  
524 corresponding Visual Minteq calculations (see section Cr ecotoxicity in Results and  
525 discussion for details). Assuming a density of 3.11 g/cm<sup>3</sup> for the amorphous Cr  
526 oxyhydroxides likely occurring in the medium, the total volume of the particles would be  
527 approx.  $1.45 \times 10^{-14}$  m<sup>3</sup>/mL. Given an approximate volume of  $5.24 \times 10^{-22}$  m<sup>3</sup> for an  
528 individual (spherical) particle with a diameter of 100 nm, a mass of Cr of 45 µg would be  
529 enough to form  $2.76 \times 10^7$  particles/mL. The presence of larger particles, unaccounted for in  
530 the previous calculation, can be invoked to explain the difference between calculations and  
531 SPC measurements.

532

### 533 *Implications for Cr hazard and risk assessment*

534 Our results show that Cr<sup>III</sup> speciation into a standardized ecotoxicological test medium for  
535 algae changes within a few hours after spiking; even when the total Cr<sup>III</sup> concentration  
536 remains comparatively stable. Similar, although less detailed, investigations on daphnids  
537 (Ponti et al. 2014) and bacteria (Bencheikh-Latmani et al. 2007) suggest that such behavior  
538 may be common in ecotoxicological media whose composition favors the formation of Cr<sup>III</sup>  
539 hydroxides. Thus, new ecotoxicological data for the hazard assessment of Cr<sup>III</sup> must be  
540 obtained by considering the recommendations originally developed for sparingly soluble  
541 substances (ISO 14442 2006) [46]. In particular, in the case of marked differences (> 20%)  
542 between nominal and actual concentration, the corresponding EC50 for Cr<sup>III</sup> must be  
543 calculated as the TWM of the dissolved concentration (OECD 2012). The excellent agreement  
544 between experimental results and thermodynamic modeling of speciation equally suggests  
545 that our observations could have a broader validity, although the kinetics of temporal  
546 speciation changes may vary across media and as a function of initial Cr<sup>III</sup> concentration.  
547 Furthermore, following our IC-ICPMS results, the question of the actual Cr<sup>III</sup> species  
548 responsible for the observed ecotoxicological effects in living organisms deserves additional  
549 study. An application of the biotic ligand model to the study of root elongation in barley

550 (Song et al. 2014) suggested  $\text{Cr}^{3+}$  and  $\text{Cr}(\text{OH})^{2+}$  as the two biologically active chemical  
551 species in controlling the effects of  $\text{Cr}^{\text{III}}$ . However, the rapid disappearance ( $< 2$  h) of ionic Cr  
552 from the test medium questions the role of such species in determining the observed algal  
553 response. One possibility is that a pulse exposure to  $60 \mu\text{g/L}$  of truly dissolved  $\text{Cr}^{\text{III}}$  at the  
554 beginning of the test can cause a 50% reduction in algal biomass after incubation over 72 h.  
555 Alternatively, the residual concentration of ionic  $\text{Cr}^{\text{III}}$  after 2 h (less than  $1 \mu\text{g/L}$ ) determines  
556 the effect observed at the end of the test or the other  $\text{Cr}^{\text{III}}$  species also contribute to the  
557 observed toxicity. The formation of some Cr-bearing (nano)particles over the course of the  
558 test also points to a possible nano-ecotoxicological dimension in the toxicity of  $\text{Cr}^{\text{III}}$  to algae.  
559 In particular, freshly formed  $\text{Cr}(\text{OH})_3$  particles can lead to the formation of “transiently  
560 available  $\text{Cr}^{\text{III}}$  species” which may be highly toxic (Bencheikh-Latmani et al. 2007). The  
561 existence of monomeric and polymeric  $\text{Cr}^{\text{III}}$  being documented in environmental samples (Hu  
562 et al. 2016), both Cr hydroxides and Cr-bearing (nano)particles may have an ecotoxicological  
563 role outside laboratory settings and deserve consideration in risk assessment for  $\text{Cr}^{\text{III}}$ .  
564 The present study does not question the high ecotoxicity of  $\text{Cr}^{\text{VI}}$ , but it adds to the growing  
565 evidence that the biological effects of  $\text{Cr}^{\text{III}}$  strongly depend on the composition of the medium  
566 used for testing and are likely underestimated (Lira-Silva et al. 2011; Ponti et al. 2014). In this  
567 context, the paradigm ‘ $\text{Cr}^{\text{VI}}$  is more toxic than  $\text{Cr}^{\text{III}}$ ’ increasingly appears as an oversimplified  
568 representation of environmental reality. In this context, more detailed studies on the  
569 speciation  $\text{Cr}^{\text{III}}$  in standardized ecotoxicological laboratory settings are highly desirable for at  
570 least three reasons. First, reduction of  $\text{Cr}^{\text{VI}}$  to  $\text{Cr}^{\text{III}}$  is a typical strategy for remediation of Cr-  
571 contaminated matrices and environments; second Cr contamination remains a serious  
572 environmental problem in several low to middle income countries whose economic activity is  
573 linked with the material demands of the current lifestyle in developed countries (Pure Earth  
574 and Green Cross 2016); third the safe perspective use of  $\text{Cr}_2\text{O}_3$  nanoparticles is linked to their  
575 intrinsic properties, but also to the possible release of soluble Cr in either redox forms (Horie  
576 et al. 2013; da Costa et al. 2016; Puerari et al. 2016). In all cases, the appropriate knowledge  
577 of the actual ecotoxicity of  $\text{Cr}^{\text{VI}}$  and  $\text{Cr}^{\text{III}}$  necessary to assess the environmental risks of Cr  
578 appears incomplete. Identifying the exact chemical species responsible for ecotoxicological  
579 effects, especially in the case of  $\text{Cr}^{\text{III}}$ , will facilitate extrapolation of laboratory findings to  
580 complex environmental realities and the associated remediation/management decision.

581  
582 *Supplemental data*— The Supplemental Data are available on the Wiley Online Library at  
583 DOI: 10.1002/etc.xxx. Details for IC-ICP-MS experiments and analytical procedures,  
584 temporal evolution of dissolved  $\text{Cr}^{\text{III}}$  and  $\text{Cr}^{\text{VI}}$  concentrations, complementary IC-ICP-MS  
585 results, optimization and details of NTA and spICP-MS procedures and additional  
586 measurements of particle size distribution and number by SPC, NTA and spICP-MS.

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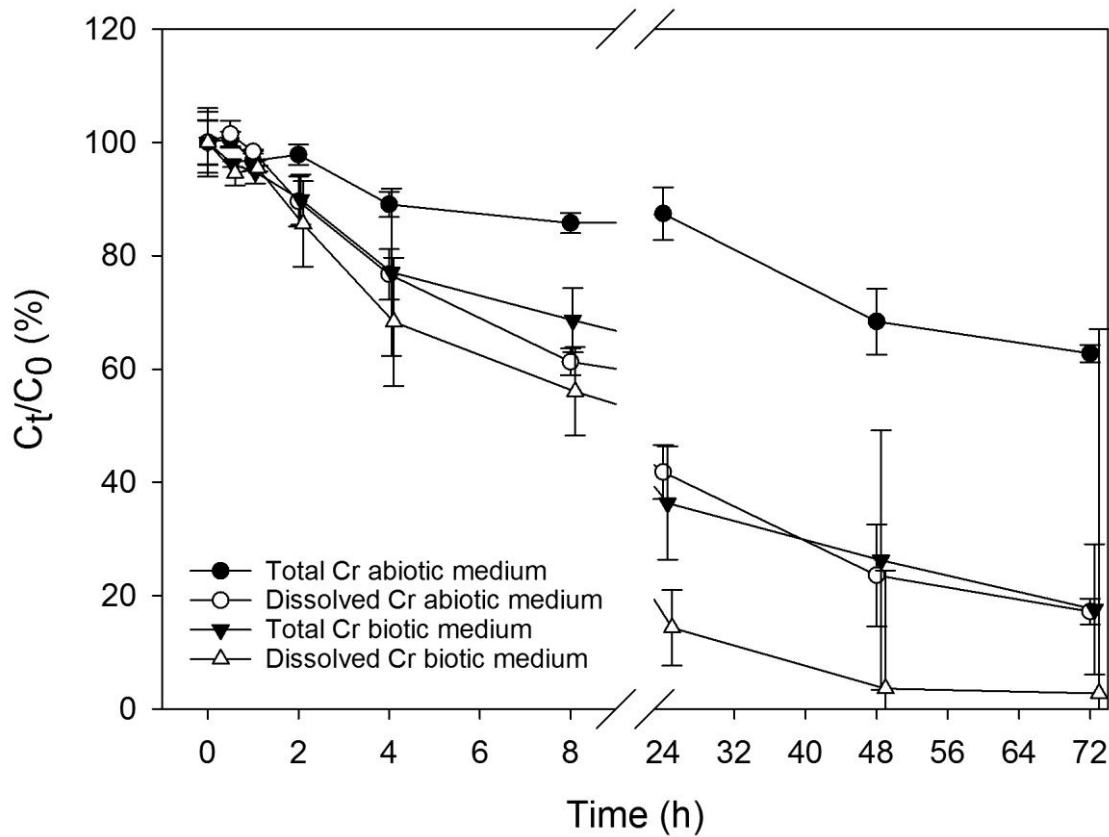
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810 **Figure 1.**

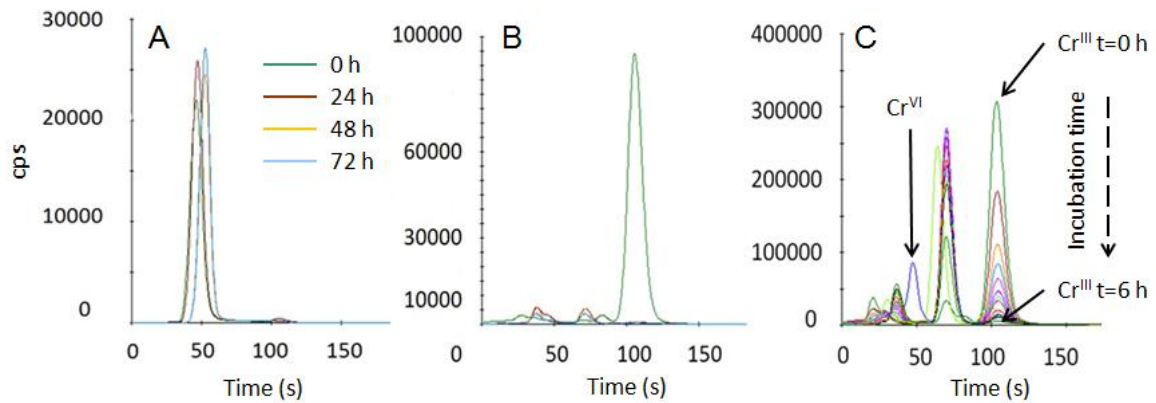


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813 **Figure 1.** Temporal evolution (expressed as percentage of the value measured at  $t = 0$ ) of total  
814 and dissolved ( $< 0.22 \mu\text{m}$ ) Cr concentrations in ISO medium amended with  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$  at an  
815 initial concentration of  $60 \mu\text{g Cr/L}$  (nominal value). Error bars indicate one standard deviation  
816 (SD). Large SD after 72 h of incubation were caused by Cr concentration decreasing to values  
817 close to the instrumental detection limit.  $C_0$  and  $C_t$  refer to total or dissolved Cr concentration  
818 measured at  $t=0$  and at the various incubation times, respectively. All data points were  
819 calculated on the basis of measured total and dissolved Cr values. The measured pH was 7.8–  
820 7.9 over the entire duration of the experiment. Data points for different incubation times were  
821 slightly nudged to visualize the corresponding error bars.

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834 **Figure 2.**

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839 **Figure 2.** Chromatograms of test medium (without added algae) at different incubation times.  
840 Panels A and B: test medium amended with 115  $\mu\text{g/L}$  of  $\text{Cr}^{\text{VI}}$  ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) (A) or 60  $\mu\text{g/L}$  of  
841  $\text{Cr}^{\text{III}}$  ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ) (B) at 0 h, 24 h, 48 h or 72 h after spiking. Panel C: temporal evolution of  
842 chromatograms of test medium containing 60  $\mu\text{g/L}$  of  $\text{Cr}^{\text{III}}$  with measurements performed  
843 every 20 min during 6 h. Note the peak of added  $\text{Cr}^{\text{VI}}$  (5  $\mu\text{g/L}$ ) at 50 s. All solutions were  
844 filtered (0.22  $\mu\text{m}$ ) immediately before analysis; cps (counts per second). Note the different  
845 scales on the y axes.

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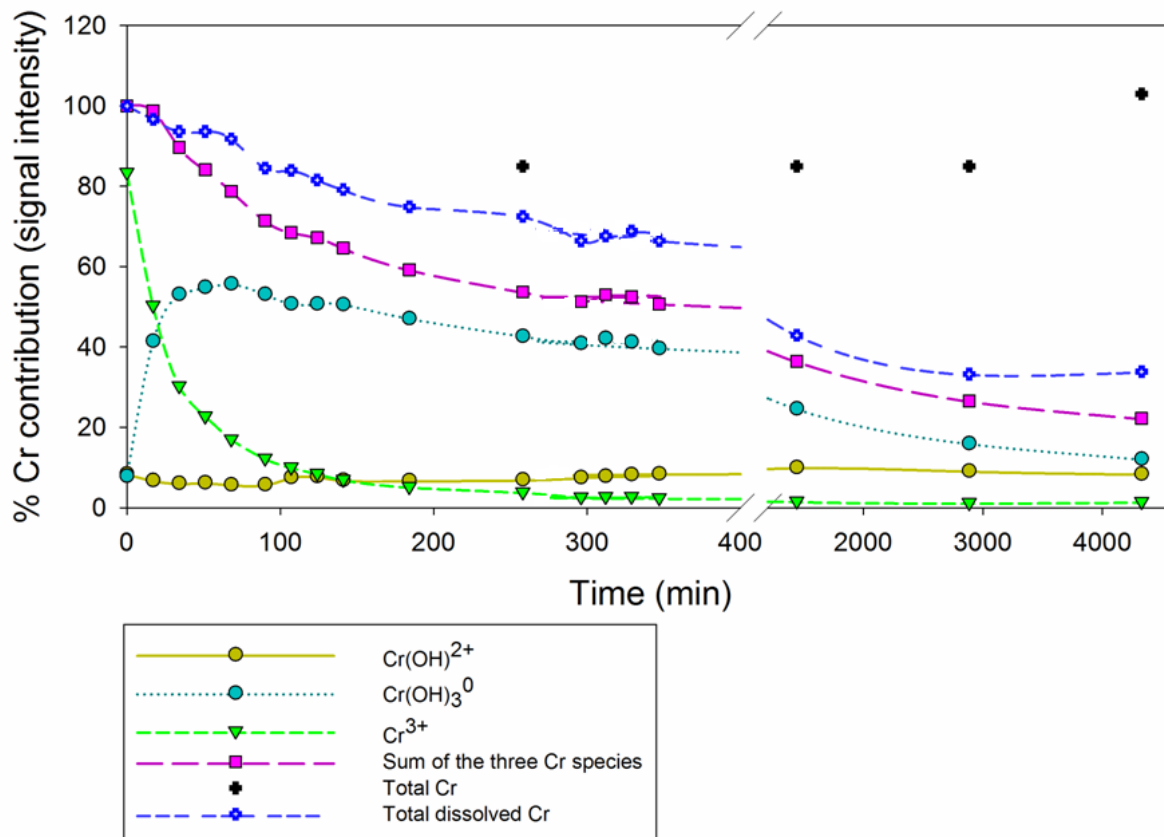
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869 **Figure 3.**

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873 **Figure 3.** Temporal evolution of the relative abundances of three Cr<sup>III</sup> species (Cr(OH)<sup>2+</sup>,  
 874 yellow circles; Cr(OH)<sub>3</sub><sup>0</sup>, turquoise circles; Cr<sup>3+</sup>, green triangles) in ISO medium spiked with  
 875 60 μg Cr/L of Cr<sup>III</sup> (CrCl<sub>3</sub>·6H<sub>2</sub>O) determined by IC-ICP-MS. The sum of the three Cr species  
 876 (pink squares) and the signals of total chromium (black crosses) and total dissolved chromium  
 877 (filtered at 0.22 μm; blue crosses) are also indicated.

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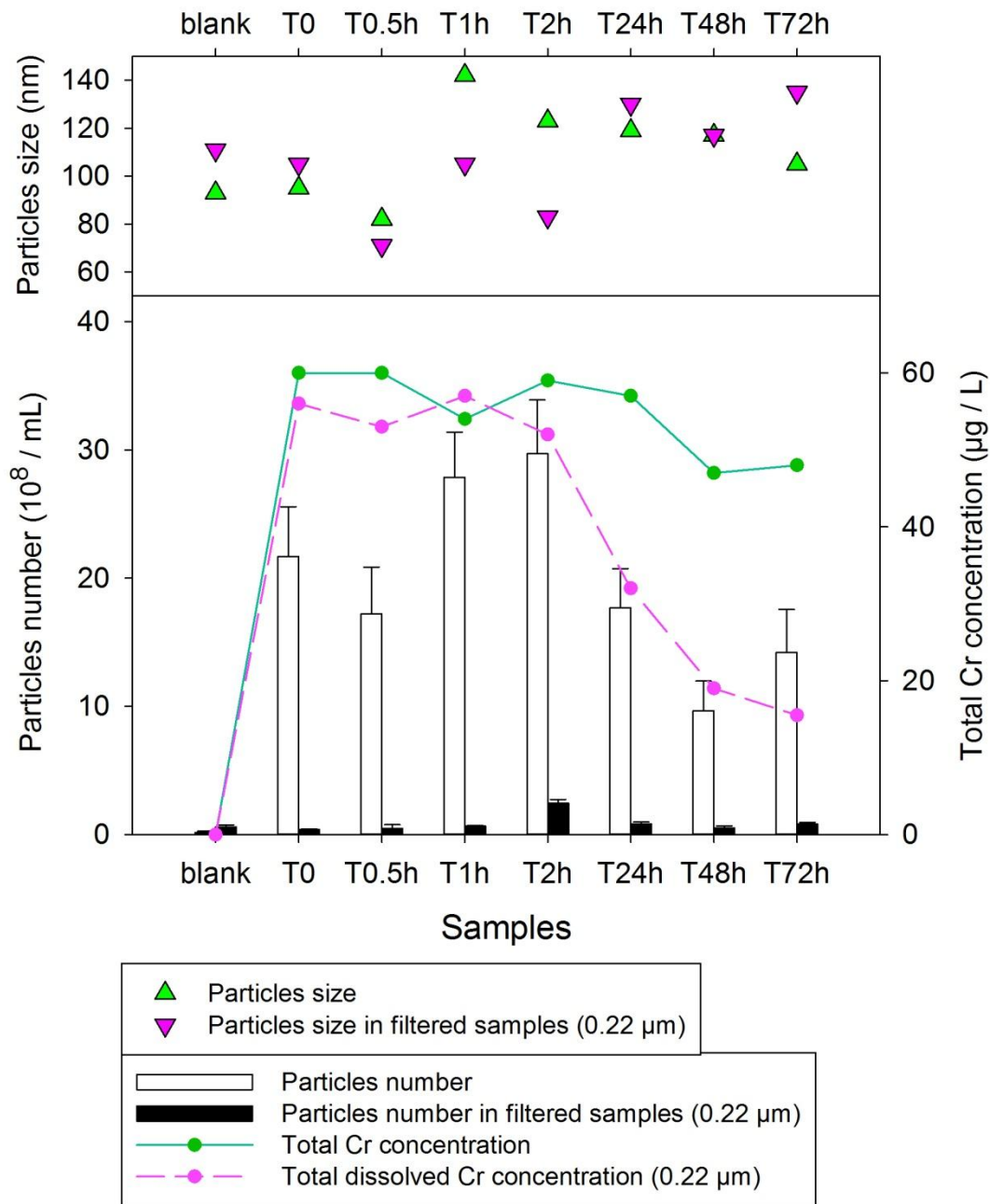
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893 **Figure 4.**

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897 **Figure 4.** Particle concentrations (bars), particle size (triangles) and Cr concentration (lines)

898 in unfiltered and filtered (0.22 µm) aliquots of ISO medium spiked (except for the sample

899 'Blank') with 60 µg/L of Cr<sup>III</sup> (CrCl<sub>3</sub> · 6H<sub>2</sub>O) at different incubation times. Particle

900 concentration and size measured by nanoparticle tracking analysis, Cr concentration measured

901 by atomic absorption spectrometry.

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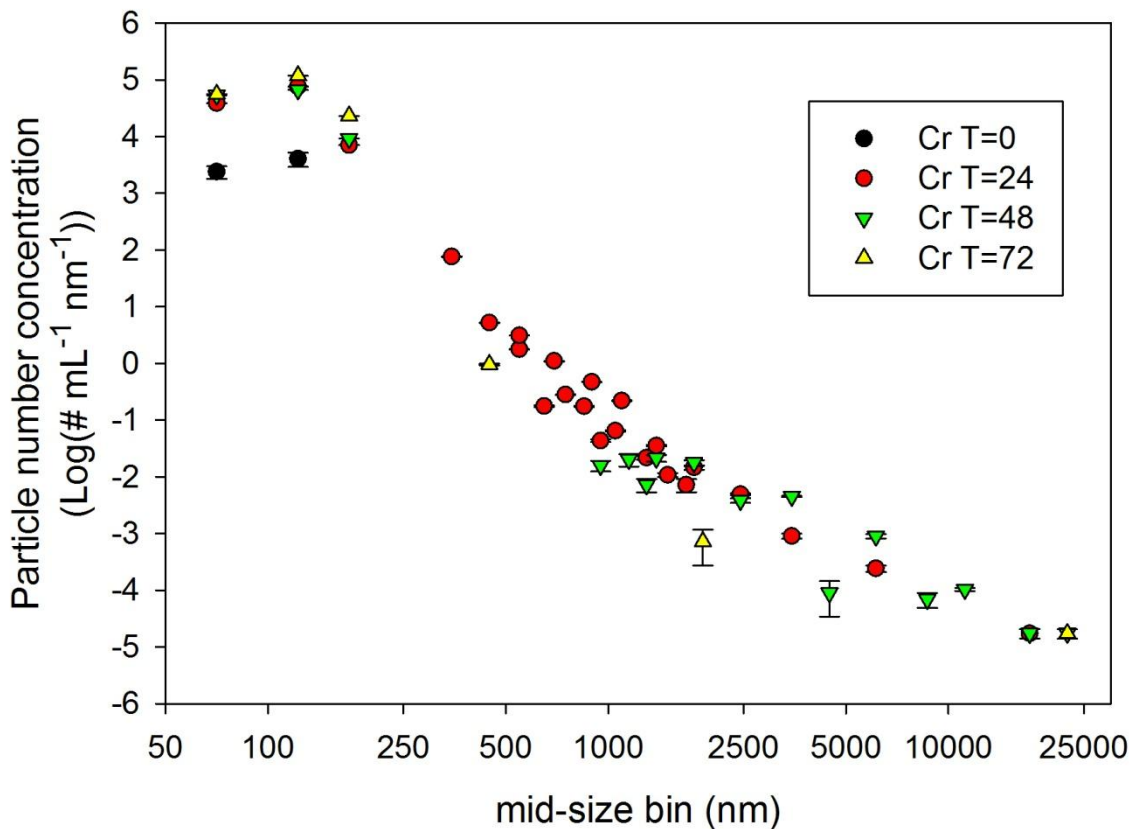
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907 **Figure 5.**



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910 **Figure 5.** Particle size distribution and number of particles (in mL<sup>-1</sup> nm<sup>-1</sup>) in test medium  
911 spiked with Cr<sup>III</sup> (CrCl<sub>3</sub>· 6H<sub>2</sub>O) after 0 (black circles), 24 (red circles), 48 (green triangles)  
912 and 72 h (yellow triangles) of incubation. All measurements were performed by Single  
913 Particle Counter (SPC) at a sample flow rate of 64 mL/min. Results for each size class have  
914 been corrected for particle concentration in ISO medium without added Cr<sup>III</sup> (see figure S7 for  
915 the corresponding comparisons). To obtain the number of particles per mL in a given size  
916 range, the values on the y-axis must be multiplied for the corresponding size range (in nm) on  
917 the x-axis.

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## Supporting information

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Chromium speciation in a standardized ecotoxicological medium:  
implications for hazard and risk assessment

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Twelve supplementary figures and three supplementary tables



967 **Contents – Supporting information :**

968 Figure S1: Experimental plan for Cr speciation experiments with Ion Chromatography ICP-MS (IC-  
969 ICP-MS)

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971 Figure S2: Temporal evolution of two Cr<sup>III</sup> initial concentrations (total dissolved) in ISO8692 medium  
972 over 72 h

973 Figure S3: Temporal evolution of nine Cr<sup>III</sup> initial concentrations (total dissolved) in ISO8692 medium  
974 over 72 h

975 Figure S4: Temporal evolution of nine Cr<sup>III</sup> initial concentrations (total) in ISO8692 medium over 72 h

976 Table S2: Measured Cr concentration at t = 0 in standard (ISO 8692) medium amended with different  
977 initial concentrations with and without filtration

978 Figure S5: Temporal evolution of Cr<sup>VI</sup> concentrations (CE<sub>50</sub> as added initial concentration) with and  
979 without filtration in biotic (presence of algae) ISO medium.

980 Figure S6: Chromatograms of standard ISO 8692 biotic (presence of algae) medium amended with  
981 Cr<sup>VI</sup> or with Cr<sup>III</sup> at different times between 0 and 72 h

982 Figure S7: Particle size distribution and number of particles determined with Single Particles Counter  
983 in ISO algal test medium with and without addition of Cr<sup>III</sup> at different incubation times

984 Figure S8: Particle size distribution and number of particles determined with Nanoparticle Tracking  
985 Analysis in ISO algal test medium with and without addition of Cr<sup>III</sup> at different incubation times

986 Figure S9: Nanoparticle Tracking Analysis results profiles before and after measurement parameters  
987 modification.

988 Figure S10: Temporal evolution of chromium concentrations with and without filtration in ISO  
989 medium amended with Cr<sup>III</sup> during 72 hours.

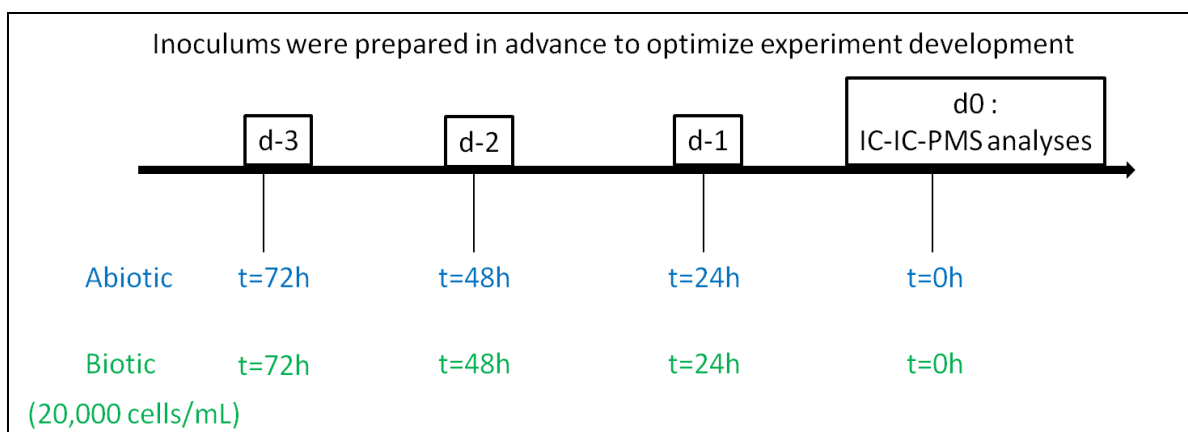
990 Table S3: Conditions and ICP-MS parameters for spICP-MS analysis.

991 Figure S11: Temporal evolution of particle number and % of total mass occurring as particle mass or  
992 Number average and mass average particle diameter.

993 Figure S12: Time-dependent changes in the distribution of the corresponding spherical diameter.

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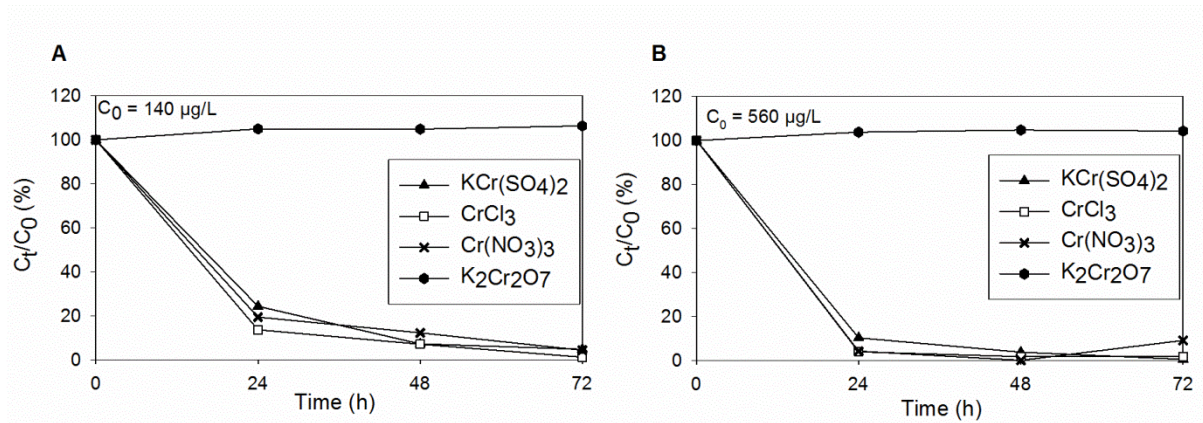


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997 Figure S1. Experimental plan for speciation experiments with Ion Chromatography ICP-MS (IC-ICP-  
 998 MS). Abiotic (absence of algae) and biotic (presence of algae) samples were prepared in advance or  
 999 immediately prior to analysis (d0). All samples were analyzed on day 0 to optimize the analyses  
 1000 course in day 0.

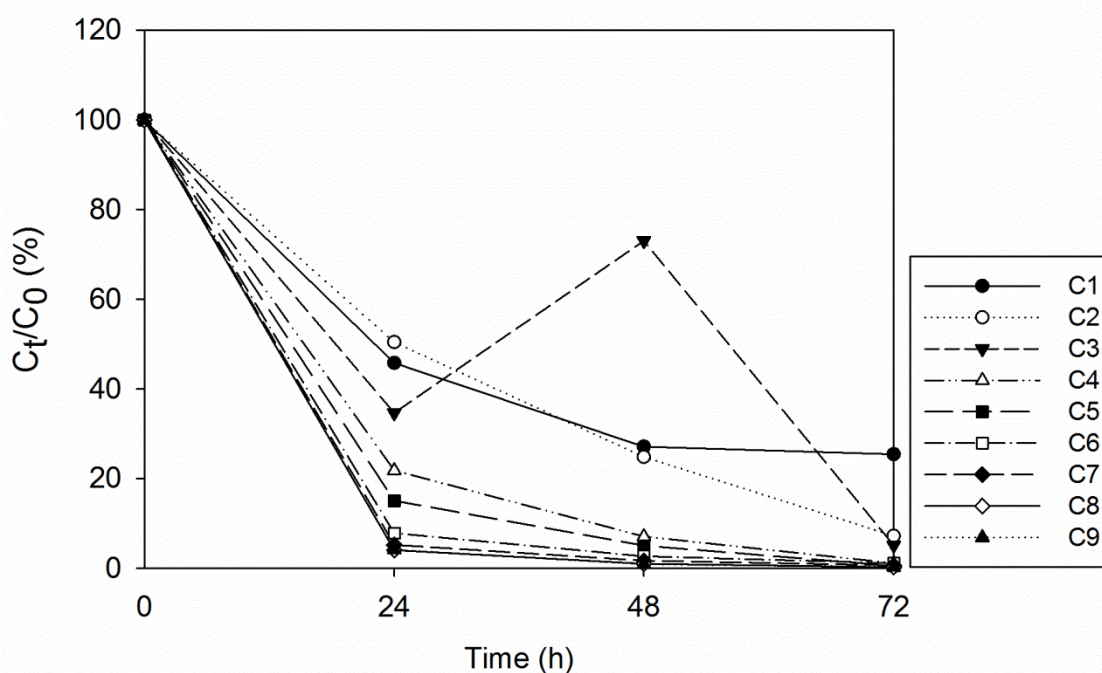
1001 Table S1: Summary of the IC-ICP-MS parameters and conditions used in Cr speciation experiments

|                          |  |
|--------------------------|--|
| <b>IC conditions</b>     |  |
| pump                     | Thermo ICS 5000+   |
| separation column        | Dionex Thermo AG7 2x50 mm  |
| column temperature       | 20 °C  |
| eluent                   | 0.4 mol/L HNO <sub>3</sub> spiked with 1µg/L of Rh, isocratic system |
| flow rate                | 500 µL/min   |
| sample volume            | 200 µL   |
| flushing volume          | 1,500 µL   |
| duration                 | 10 min   |
| <b>ICP-MS parameters</b> |  |
| ICP-MS                   | Thermo XII series  |
| nebulizer                | PFA-LC   |
| gas flow                 | 0.87 L/min   |
| plasma power             | 1,500 W  |
| plasma gas flow          | 13.5 L/min   |
| auxiliary gas flow       | 0.77 L/min   |
| detector mode            | pulse counting   |
| KED                      | He/H <sub>2</sub>  |
| CCT – KED flow rate      | 3.5 mL/min – 3 V   |
| <b>Data acquisition</b>  |  |
| ions (m/z)               | <sup>52</sup> Cr, <sup>53</sup> Cr, <sup>103</sup> Rh                |
| measurement unit         | counts   |
| sweeps/reading           | 1  |
| replicates               | 1  |
| dwel time                | 100 ms   |
| integration time         | 600,000 ms   |



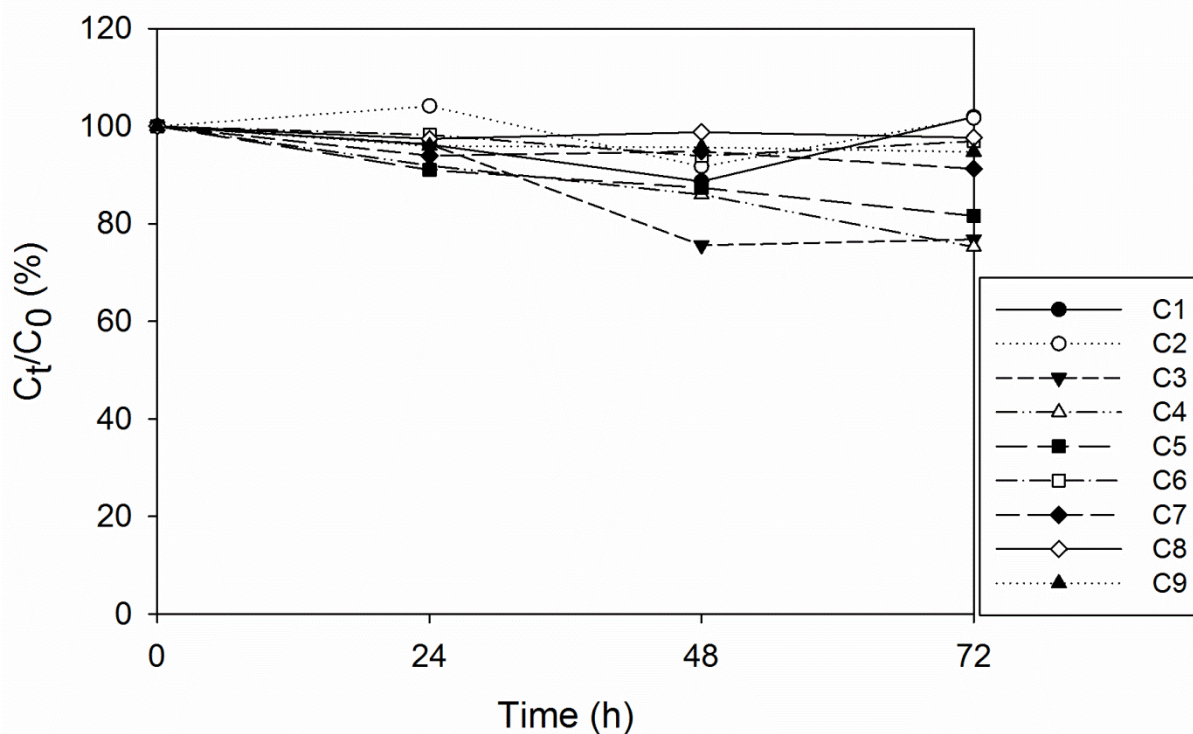
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 1003 Figure S2. Temporal evolution (expressed as a percentage of the value measured at t=0) of total  
 1004 dissolved Cr concentrations (filtered at 0.22 µm) in standard (ISO8692) medium amended with Cr<sup>III</sup>  
 1005 (CrCl<sub>3</sub>·6H<sub>2</sub>O (square) or Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O (cross) or KCr(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O (triangle)) or Cr<sup>VI</sup> (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>  
 1006 (circle)) during 72 hours. Initial concentration of each salt was 140 µg/L (A) or 560 µg/L (B).

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 1024 Figure S3. Temporal evolution (expressed as a percentage of the value measured at t=0) of total  
 1025 dissolved Cr concentrations (filtered at 0.22  $\mu\text{m}$ ) in standard (ISO 8692) medium amended with initial  
 1026 total concentrations of  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$  between 20 (C1) and 7000 (C9)  $\mu\text{g/L}$  (see Table S2 for the full  
 1027 compilation of the measured values at t = 0). Measurements were done in triplicate for C1, C2, C4 and  
 1028 C9 after 0 and 48 h of incubation. The relative standard deviations (RSD) for these measurements  
 1029 were usually below 10 % except for C1 at 48 h (RSD = 70%) and C2 at 48 h (RSD = 40%) and C3 at  
 1030 48 h (RSD = 20%). The high RSD were obtained for analytical concentrations close to the  
 1031 instrumental quantification limit for FAAS. Also note that, with increasing concentrations, total  
 1032 dissolved Cr levels were lower than the corresponding total ones already at t = 0 (see Table S2).

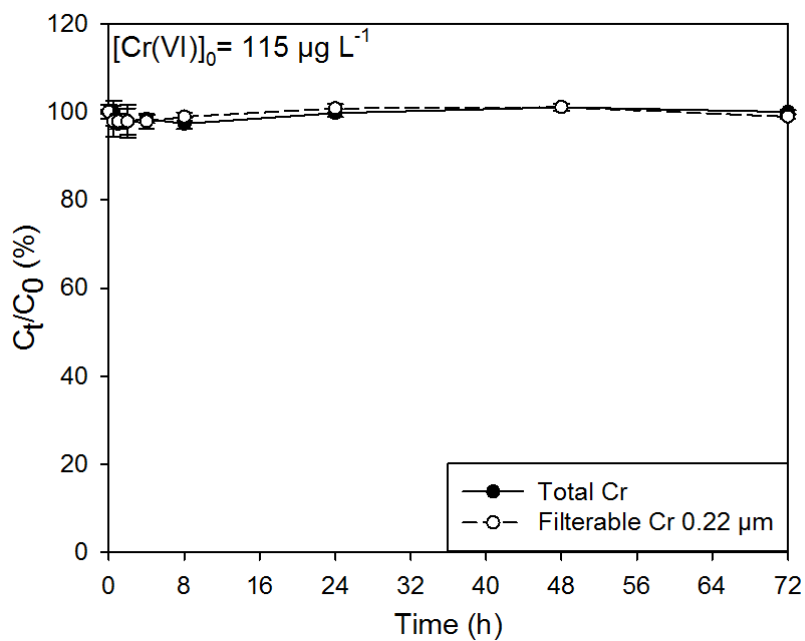
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 1047 Figure S4. Temporal evolution (expressed as a percentage of the value measured at  $t = 0$ ) of total Cr  
 1048 concentrations in standard (ISO 8692) medium amended with initial concentrations of  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$   
 1049 between 20 (C1) and 7000 (C9)  $\mu\text{g/L}$  (see Table S2 for the full compilation of the measured values at  $t$   
 1050  $= 0$ ). Measurements were done in triplicate for C1, C2, C4 and C9 after 0 and 48 h of incubation. The  
 1051 relative standard deviations (RSD) for these measurements were usually below 10 % except for C1 at  
 1052 48 h (RSD = 20%) and C2 at 48 h (RSD = 15%).

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 1055 Table S2. Measured Cr concentration at  $t = 0$  in standard (ISO 8692) medium amended with different  
 1056 initial concentrations (C1–C9) of  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$  with and without filtration at  $0.22 \mu\text{m}$ . All values are in  
 1057  $\mu\text{g/L}$  of Cr.

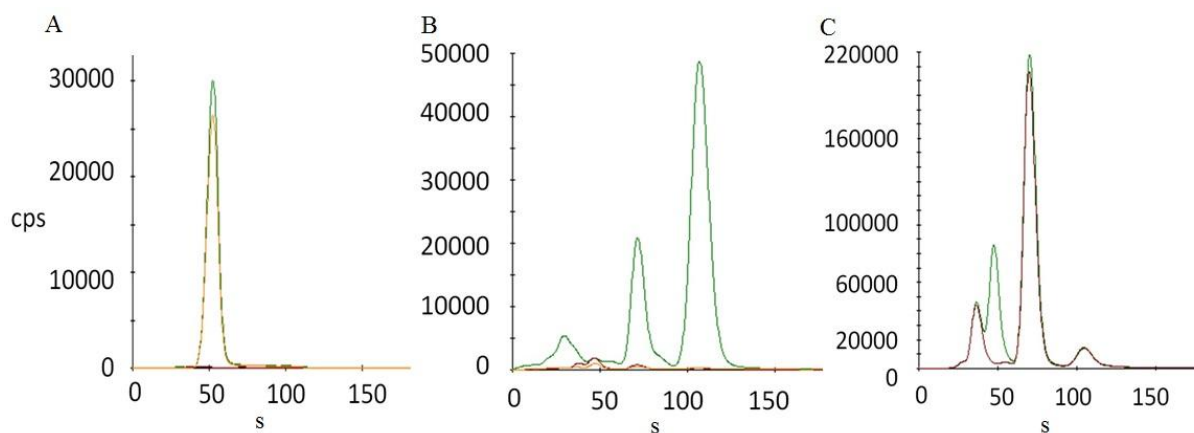
|    | Measured total concentration at $t = 0$ | Measured filterable ( $0.22 \mu\text{m}$ ) concentration at $t = 0$ |
|----|---|---|
| C1 | 18                                      | 20  |
| C2 | 40                                      | 42  |
| C3 | 82                                      | 78  |
| C4 | 186                                     | 179   |
| C5 | 436                                     | 379   |
| C6 | 789                                     | 717   |
| C7 | 1745                                    | 1477  |
| C8 | 3596                                    | 2700  |
| C9 | 6877                                    | 5489  |



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 1060 Figure S5. Temporal evolution (expressed as a percentage of the values measured at  $t = 0$ ) of Cr  
 1061 concentrations (total Cr concentrations (black circles) and total dissolved Cr concentrations (filtered at  
 1062 0.22  $\mu\text{m}$ ) (white circles) in biotic (presence of algae) ISO medium amended with  $\text{Cr}^{\text{VI}}$  ( $\text{K}_2\text{Cr}_2\text{O}_7$ )  
 1063 during 72 hours. Initial Cr concentration is 115  $\mu\text{g/L}$ .

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1085 Figure S6. Chromatograms of standard ISO 8692 biotic (presence of algae, initial density ~ 20,000  
1086 cell/mL) medium amended with 115  $\mu\text{g/L}$  of  $\text{Cr}^{\text{VI}}$  ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) (A) or with 60  $\mu\text{g/L}$  of  $\text{Cr}^{\text{III}}$  ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ )  
1087 (B) at 0 h (green), 24 h (brown), 48 h (orange) or 72 h (blue) after spiking. Chromatograms of standard  
1088 ISO 8692 medium containing 60  $\mu\text{g/L}$  of  $\text{Cr}^{\text{III}}$  ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ) during 24 h (brown), and then amended  
1089 with  $\text{Cr}^{\text{VI}}$  ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) (green) (C). All solutions were filtered (0.22 $\mu\text{m}$ ) immediately before analysis; cps  
1090 (counts per second).

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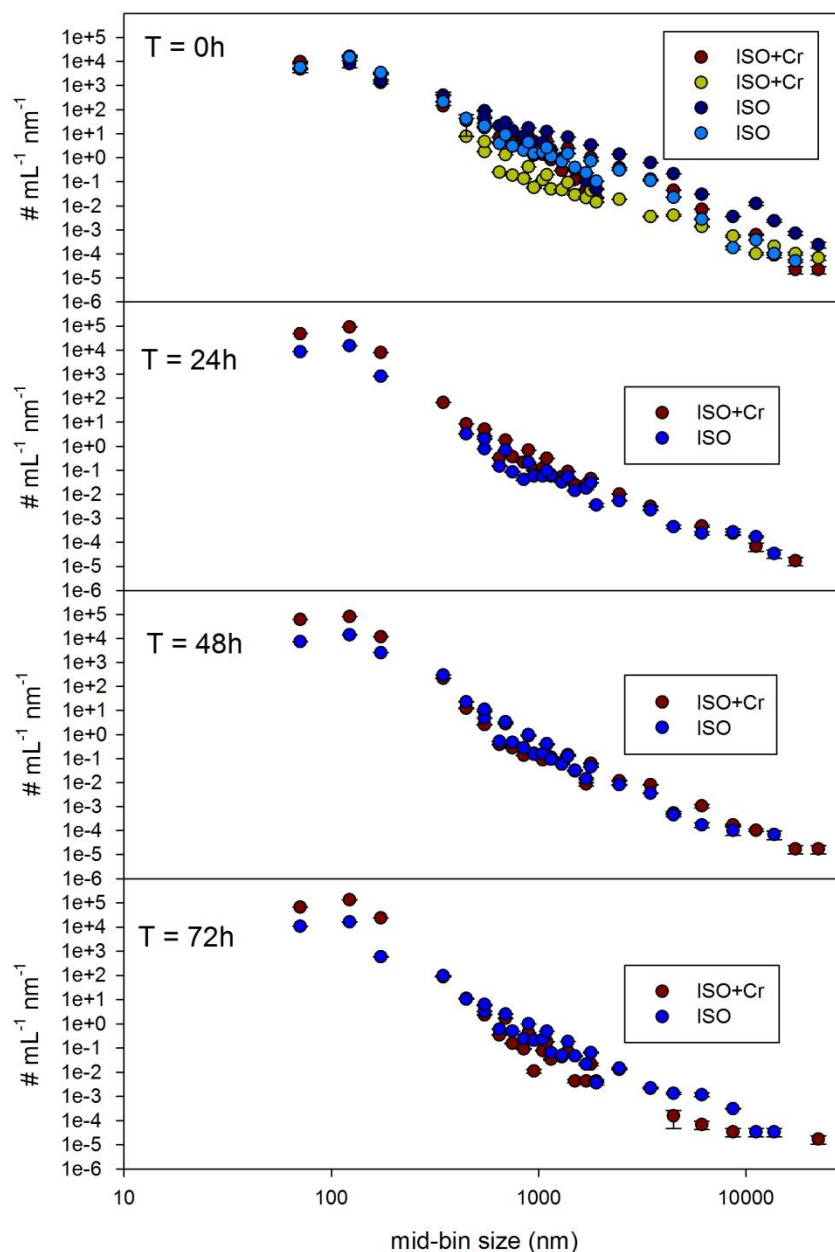
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 1105 Figure S7. Particle size distribution and number of particles (in  $\text{mL}^{-1} \text{nm}^{-1}$ ) determined with Single  
 1106 Particle Counter (SPC) measurements in ISO algal test medium with and without addition of  $70 \mu\text{g/L}$   
 1107 of  $\text{Cr}^{\text{III}}$  (added as  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ) at different incubation times. To obtain the number of particles per mL  
 1108 in a given size range, the values on the y-axis must be multiplied for the corresponding average size  
 1109 value (in nm) on the x-axis.  
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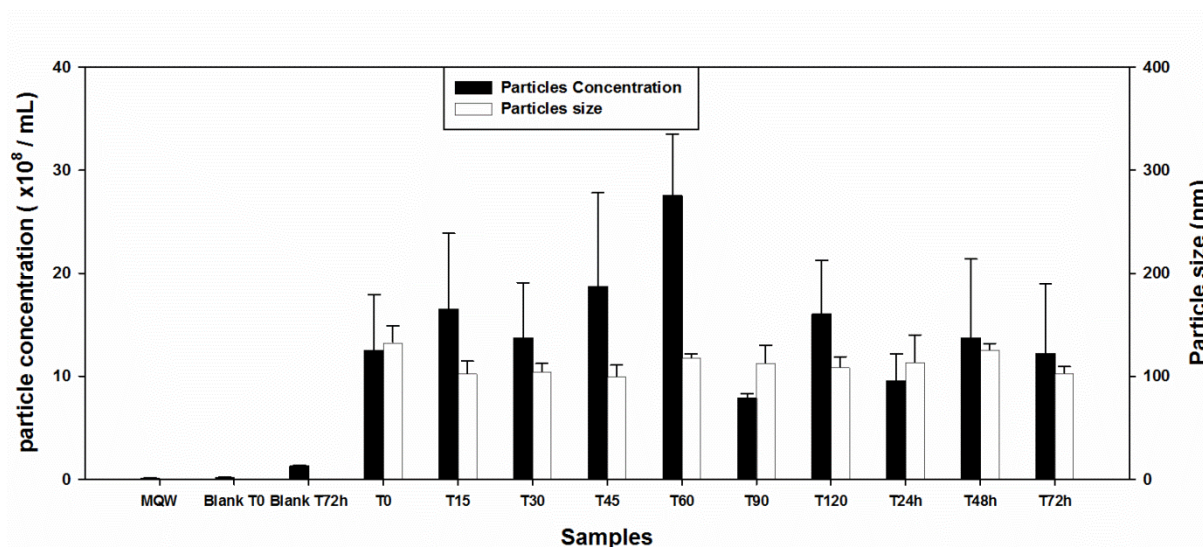


1113 **Optimization of operating conditions for Nanoparticle Tracking Analysis with**  
1114 **Nanosight NS500 instrument (Malvern instruments Ltd, UK)**

1115 In a preliminary experiment, aliquots of test medium spiked with 0, 17, 35, 70, 140 and 280  
1116  $\mu\text{g/L}$ , partly covering the concentration range used in toxicity test, were analyzed by NTA. No  
1117 particles were observed in aliquots spiked with 17  $\mu\text{g/L}$   $\text{Cr}^{\text{III}}$  and too many particles were  
1118 detected in aliquots spiked with 140  $\mu\text{g/L}$  and 280  $\mu\text{g/L}$   $\text{Cr}^{\text{III}}$ . Particle concentrations in the  
1119 range  $1\text{--}3 \times 10^9$  particles/mL were observed in solutions containing 35 and 70  $\mu\text{g/L}$   $\text{Cr}^{\text{III}}$ . The  
1120 latter concentration, closed to the observed 72h EC50 for  $\text{Cr}^{\text{III}}$ , was chosen for detailed  
1121 analysis after 1, 15, 30, 45, 60, 90 and 120 min, and 24, 48 and 72 h of incubation.

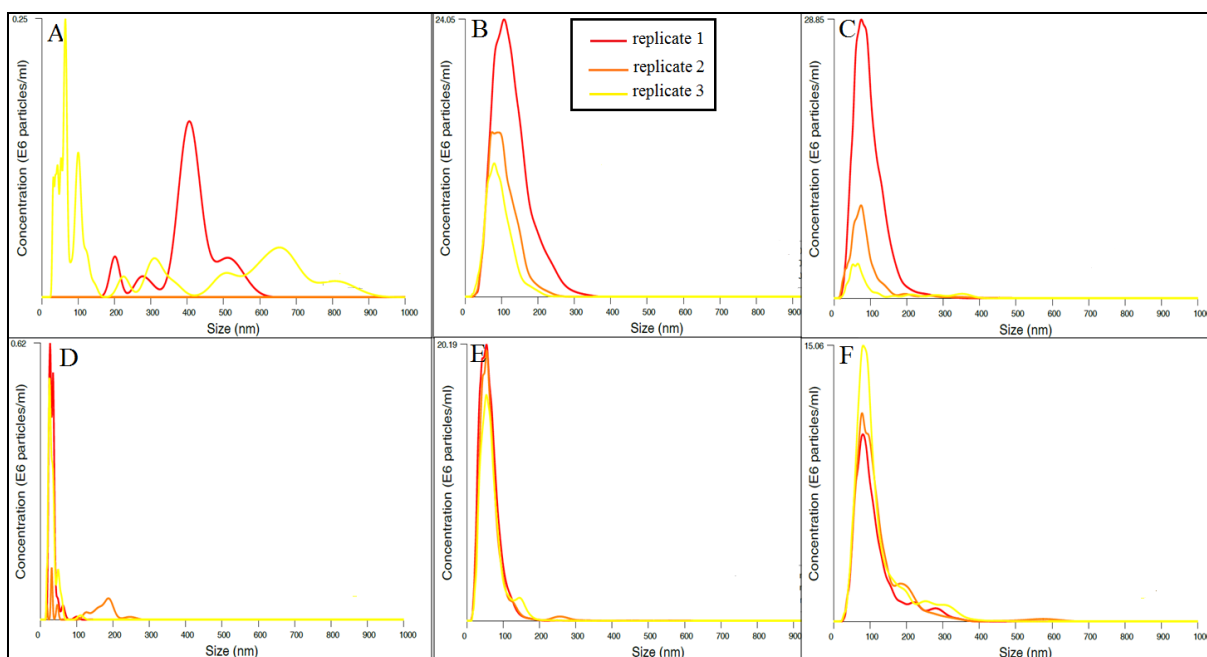
1122 No particles were detected in the ultrapure water (MQW) used to prepare the test medium.  
1123 Particle concentration in the blank sample (freshly prepared ISO medium) was low ( $0.14 \pm$   
1124  $0.07 \times 10^8$  particles/mL) and particle size distribution (PSD) covered a large spectrum  
1125 between 0 and 900 nm (Figure S9). In ISO medium spiked with  $\text{Cr}^{\text{III}}$  and regardless of the  
1126 incubation time, particle concentrations ranged between  $7.85 \times 10^8$  and  $27.5 \times 10^8$  particles/mL  
1127 with relative standard deviations (RSD) between 6 and 59 % (Figure S8 and S9). Particles  
1128 detected by NTA had a mean size between 100 and 135 nm with RSD between 1% and 12%  
1129 (Figure S9). The high RSD of some particle concentration measurements were caused by the  
1130 systematic decrease in particle number between the first and the third replicate measurement  
1131 (Figure S9 A, B and C). The total (unfiltered) Cr concentration was stable over 72 h and  
1132 ranged between 62  $\mu\text{g/L}$  at  $t=0$  and 66  $\mu\text{g/L}$  72 h after spiking. However, the total filterable Cr  
1133 concentration decreased over time from 60  $\mu\text{g/L}$  at  $t=0$  to 16  $\mu\text{g/L}$  after 72 h (Figure S10).  
1134 Examination of the videos recorded during NTA showed that the number of particles visible  
1135 in the field of the camera was stable during data acquisition for the first replicate, but  
1136 increased with time for replicates two and three.

1137 Measurement parameters were modified by adding a waiting step of 60 s before data  
1138 acquisition for replicates two and three and RSD on particle concentrations was reduced to 1–  
1139 11% (Figure S9 D, E and F).



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1141 Figure S8. Particle concentration (black bars) and size (white bars) measured by nanoparticles tracking  
1142 analysis (NTA) in standard (ISO 8692) medium spiked or not with 70  $\mu\text{g/L}$  of  $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$  for  
1143 different incubation time between 0 and 120 min and after 24, 48 and 72 h.

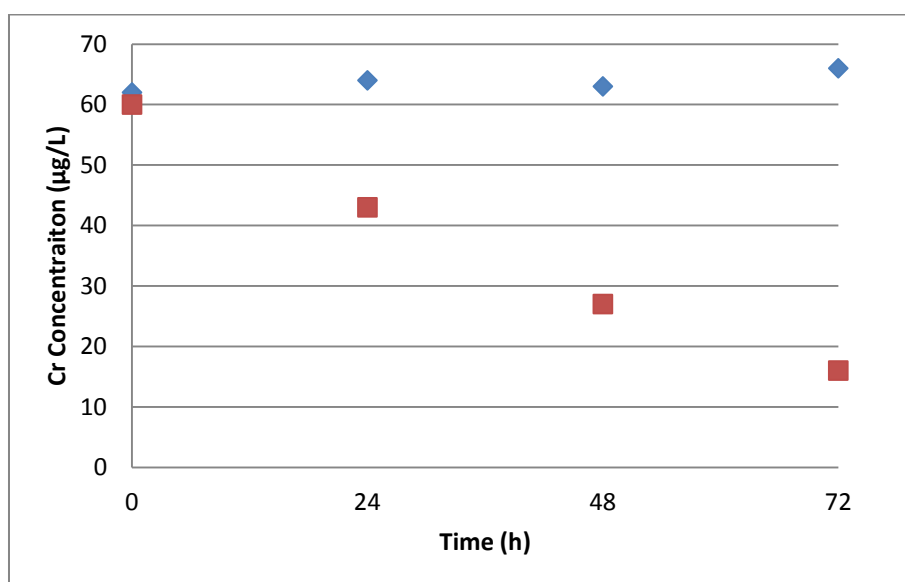
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1155 Figure S9. Comparison of reproducibility of results profiles before (A, B and C) and after (D, E and F)  
 1156 measurement parameters modification by adding a waiting step of 60 s before data acquisition for  
 1157 replicates 2 and 3. This edit to the experimental procedure aimed to harmonize the waiting time before  
 1158 each replicate measurement; i.e. to increase the waiting time for replicates two and three from 5  
 1159 seconds to 60 s. This experiment was realized in ISO test medium spiked with 60  $\mu\text{g/L}$  of  $\text{Cr}^{\text{III}}$ ,  
 1160 corresponding to the previously determined EC50 at 72 h

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1163 Figure S10. Temporal evolution of Cr concentrations in  $\mu\text{g/L}$  (total Cr concentrations (blue squares),  
 1164 and filterable Cr concentrations (0.22  $\mu\text{m}$ ) (red squares) in ISO medium amended with  $\text{Cr}^{\text{III}}$   
 1165 ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ) during 72 hours. Initial nominal Cr concentration is 70  $\mu\text{g/L}$ .

1166 **Corresponding spherical size and number concentration determination using single**  
1167 **particle ICP-MS (spICP-MS) analysis using the Nexion 350 D**

1168 When nanoparticles arrive in the ICP-MS plasma they are turned into ions thus forming a  
1169 cloud of ions. Truly dissolved ions are homogeneously distributed in solution and when  
1170 injected into the plasma thus cause a continuous, yet fluctuating, stream of ions to be detected  
1171 by the detector. Nanoparticles can thus be distinguished from dissolved ions as the former  
1172 cause intensive peaks in the detected time dependent signal, each time a cloud of ions from a  
1173 nanoparticle arrives at the detector. The intensity of this peak can be calculated into some  
1174 measure of size, provided that a composition and shape are assumed for the particles (Table  
1175 S3). Only one isotope ( $^{52}\text{Cr}$ ) was measured simultaneously, whereas any Cr-containing  
1176 particles formed because of hydrolysis most likely also contained oxygen atoms and protons.

1177 No collision cell gas was used to remove  $^{40}\text{Ar}^{12}\text{C}$  interferences on the  $^{52}\text{Cr}$  signal. Hence, a  
1178 continuous background signal was recorded, not consisting of  $^{52}\text{Cr}$  but of the  $^{40}\text{Ar}^{12}\text{C}$   
1179 interference. However, by using short dwell times, the intensity of any background signal  
1180 reduces proportionally more than the short but intense nanoparticle signals [1]. It was thus  
1181 hypothesized that better size detection limits could be obtained by using a relatively small  
1182 dwell time, but still measuring in standard mode, where the sensitivity towards the most  
1183 abundant Cr isotope ( $^{52}\text{Cr}$ ) is highest. The used dwell time of 50  $\mu\text{s}$  and the absence of settling  
1184 time cause nanoparticle events to be spread over multiple subsequent measurement events that  
1185 need to be integrated separately for each event. Moreover, distinguishing non-dissolved from  
1186 dissolved signals was needed, because interferences still caused a relatively high background  
1187 signal. Both requirements were achieved using a previously published method [2]. This  
1188 method calculates an intensity cut-off value based on a number of times the standard  
1189 deviation of the background signal. Integrated peak surfaces below this cut-off value are  
1190 considered to stem from dissolved events and are not taken into account when recalculating

1191 the average signal and the standard deviation of the background signal. The method converges  
1192 when no new particle events are identified and the particle size distribution is then calculated  
1193 from the particle events intensities. Such a single cut-off value is known to lead to both false  
1194 positives (peaks mistakenly considered particle events) and false negatives (peaks mistakenly  
1195 considered dissolved events) [3]. The goal of the spICP-MS measurement was to verify the  
1196 hypothesis of the presence of Cr-containing particles in the samples. Hence, a high and thus  
1197 conservative number of standard deviations (5) was used to minimize false positives, rather  
1198 than to minimize false negatives [4].

1199 The calculations were realized with a software called Nanocount, which is freely available at  
1200 <http://blogg.slu.se/Nanocount>.

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1211 Table S3: Conditions and ICP-MS parameters for spICP-MS analysis

| <b>ICP-MS parameters</b>                              |                         |
|---|-------------------------|
| ICP-MS  | Nexion 350D             |
| nebulizer   | GE Micromist            |
| gas flow  | 0.87 L/min              |
| plasma power  | 1,500 W                 |
| plasma gas flow                                       | 13.5 L/min              |
| auxiliary gas flow                                    | 0.77 L/min              |
| detector mode   | pulse counting          |
| KED   | Standard                |
| <b>Data acquisition</b>                               |                         |
| ions (m/z)  | <sup>52</sup> Cr        |
| measurement unit                                      | counts                  |
| sweeps/reading  | 10 <sup>6</sup>         |
| replicates  | 1                       |
| dwelt time  | 0.05 ms                 |
| integration time                                      | 50,000 ms               |
| <b>Data treatment</b>                                 |                         |
| Integration method                                    | Fixed window            |
| Peak integration time                                 | 20 ms                   |
| Minimum cluster size                                  | 4 ions                  |
| Signal discrimination method                          | Outlier analysis        |
| Number of standard deviations for cut-off calculation | 5                       |
| Assumed composition                                   | Cr(OH) <sub>3</sub>     |
| Assumed density                                       | 3.11 g cm <sup>-3</sup> |
| Assumed shape   | sphere                  |

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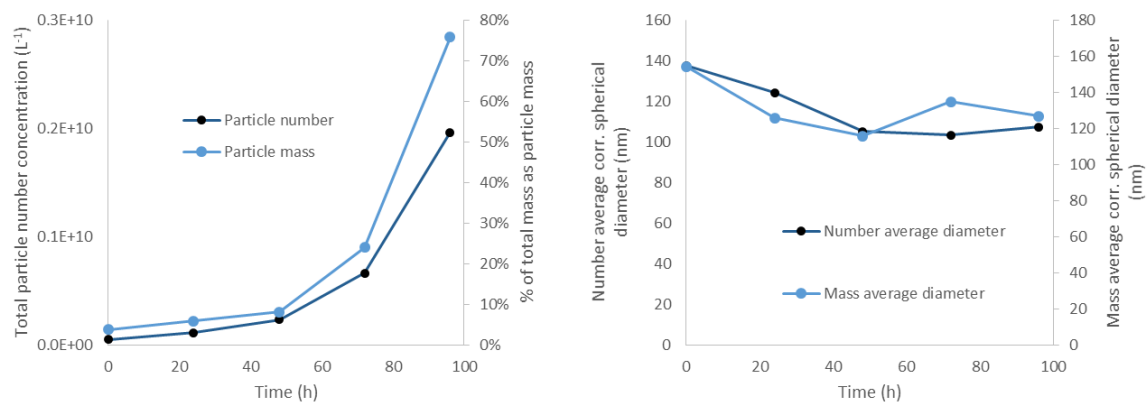
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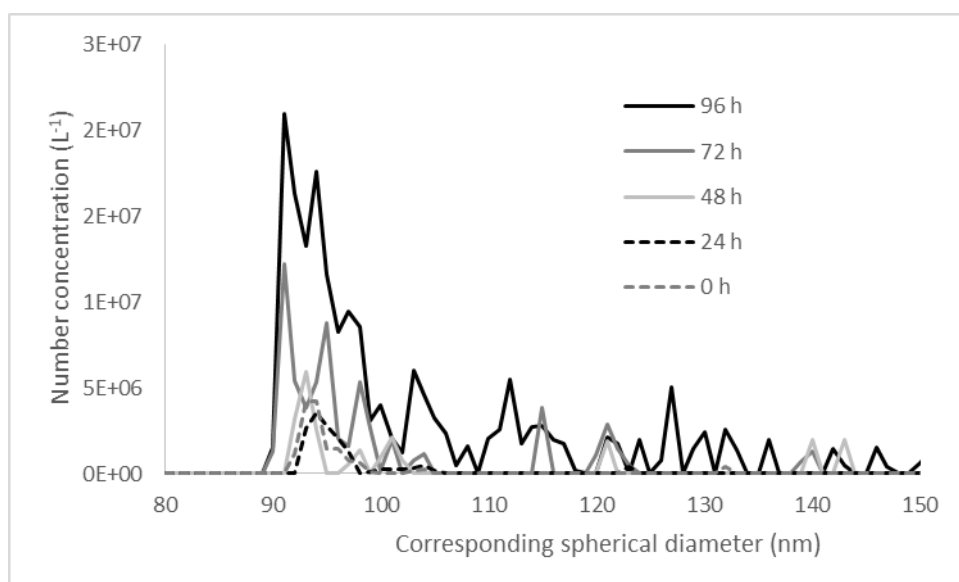
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 1220 Figure S11. Temporal evolution of particle number and % of total mass occurring as particle mass  
 1221 (left) or number average and mass average particle diameter (right). The diameters are corresponding  
 1222 spherical diameters calculated using the setting in Table S3.  
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 1225 Figure S12. Temporal evolution in the distribution of the corresponding spherical diameter. The  
 1226 diameters are corresponding to spherical diameters calculated using the setting in Table S3.  
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1234 **References**

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