Temporal Decrease of Trivalent Chromium Concentration in a Standardized Algal Culture Medium: Experimental Results and Implications for Toxicity Evaluation

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Abstract The fate of two trivalent chromium salts (nitrate and chloride) in ISO algal culture medium was followed over 72 h; i.e., the typical duration of algal toxicity tests. Fifty percent of the initial Cr spikes was lost from the solutions by 24 h, with losses up to 90% after 72 h. Monitoring of the temporal variability of Cr(III) concentrations in algal culture media appears necessary to better characterize the toxicity of trivalent chromium to algae.

Keywords Trivalent chromium · Algal ISO medium · Chromium toxicity · Chromium speciation

In surface waters, chromium occurs mainly in two oxidation states, namely Cr(III) and Cr(VI), and there is a general consensus that Cr(III) is less toxic than Cr(VI) for aquatic organisms (IUCLID 1999; Pawlisz et al. 1997; Stoecker 2004). In the case of freshwater algae, this consensus relies on a rather small set of data. A "Quick Database Query" performed with the AQUIRE database of the US EPA (http://www.epa.gov/med/Prods_Pubs/ecotox. htm) using "chromium" and "alga" as keywords and covering the period 1915–2003 (research performed on July, 25th 2006) yielded 75 records; compared with over a thousand records for Cu, 700 for Cd, 600 for Zn, and 450 for Pb. Similarly, recent comprehensive reports on risk assessment of Cr(III) (INERIS 2005; Munn et al. 2005; Pawlisz et al. 1997) have established guidelines for freshwater algae on the basis of a limited number of studies (Den Dooren 1965; Greene et al. 1988; IUCLID 1999; Meisch and Schmitt-Beckmann 1979; Turbak et al. 1986).

In these studies, the concentrations of Cr(III) in the exposure solutions were measured only at the beginning of the experiments or not at all. The ECs50 (Effect Concentrations affecting 50% of the exposed algae) or NOECs (No Observable Effect Concentrations) reported in such studies are two to three orders of magnitude higher than the Cr(III) theoretical solubility limit: about 5 μg/L in pure water for pH 7–11 (Rai et al. 1989). In this pH range, Cr(III) concentration is controlled by the formation of solid phases of low solubility. The solubility of Cr(III) increases to about 50 μg/L at pH 7.6–8.2 in the presence of 0.05 M of P (i.e., 1,000-fold the typical concentration in algal media) or decreases below 0.5 μg/L in the presence of Fe(III) for similar pH values (Rai et al. 2004; Sass and Rai 1987). At present no determination of Cr(III) solubility has been carried out in complex algal media, but the available literature suggests that formation and precipitation of insoluble Cr solid phases may occur in such media. A more detailed experimental verification of the stability of Cr(III) solutions prepared in algal media is necessary to ensure that the toxicity of Cr(III) to algae is not underestimated.

In this study, the behavior of two Cr(III) salts (nitrate and chloride) in standard algal medium ISO 8692 (ISO 2004), hereafter ISO medium, has been examined over a

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period of 72 h (corresponding to the typical duration of an algal test). Both salts are used in standard toxicity testing and require separate investigation, because they may form different chromium species in aqueous solutions. The possible implications of the results of this study for the understanding of Cr(III) toxicity and the related risk assessment are highlighted and discussed.

Materials and Methods

Stock solutions of Cr(III) (2.38 g/L) were prepared in ultrapure water (DirectQ 5 system, Millipore, Lausanne, Switzerland) using analytical grade chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$; purity > 98%) and chloride ($CrCl_3 \cdot 6H_2O$; purity > 96%); both from VWR. Four solutions of each salt (C1 = 2,380; C2 = 476; C3 = 95; and C4 = 19 μg/L of chromium) were prepared by appropriate dilution in ISO medium immediately prior to use. Concentrations C1–C4 cover the range of exposure used in algal toxicity tests and the range of EC50 and NOEC values reported in the literature.

Aliquots of solutions were transferred into sterilized, polystyrene microplates (96 wells; 300 μL per well). The external wells were filled with ultrapure water to minimize evaporative losses which, assuming uniform evaporation, were approx. 3% per well by weight. Chromium solutions were recovered after 0, 24, 48, and 72 h and acidified at 1% with concentrated nitric acid (VWR, Suprapur). Care was taken not to mix the solutions before their retrieval from the wells and only 200 μL of solution were recovered from each well. This procedure should ensure that any precipitate deposited on the bottom of the wells was not recovered for analysis, although freshly formed colloidal particles could have been sampled in this way. Between recoveries, microplates were kept at 24 ± 1°C under continuous light (5,000 lux), simulating typical conditions for algal tests. Experiments were performed in triplicate, unless otherwise indicated.

With the exception of microplates, all the material was washed with 10% nitric acid (VWR, proanalysis) for at least 24 h and thoroughly rinsed with ultrapure water. Preliminary tests showed that no Cr contamination was caused by the microplates when they were used as received from the supplier. Control experiments for possible Cr losses by adsorption onto microplates' walls included (a) 72 h-experiments in ISO medium using acid-washed microplates; (b) short-term experiments in ISO medium (measurements of Cr concentrations at t = 0, 1, 3, 5, and 24 h); and (c) 72 h-experiments in ultrapure water. Reaction half-times for adsorption phenomena are typically in the order of seconds to hours (Langmuir 1997) and Cr losses by adsorption should already be visible in the short-term experiments (b). Experiment (c) allowed to check for Cr adsorption in the pH range 4.2–6.0 (experimentally measured values); i.e., in a situation where the formation of insoluble chromium hydroxides should be limited or non existent. For each experiment and for each concentration tested, the statistical significance of concentration differences among sampling times was assessed by one-way ANOVA (followed by pairwise multiple comparison; Holm-Sidak method) or by t-test (see Tables 1, 2, 3 and 5–7 for details).

Total chromium concentrations were determined by ICP-MS at m/z 53 using external calibration and internal standard correction. Immediately prior to analysis, sample aliquots were diluted 51-, 26-, 21-, and 6-fold for C1, C2, C3, and C4, respectively. The dilutions were necessary to avoid excessive isobaric interference by chloride ions present in the ISO medium on Cr measurements. Unspiked aliquots of ISO medium analyzed at the same dilution factors yielded chromium concentrations below detection limits (mean ± 1 standard deviation = 0.04 ± 0.02 μg/L for 10 independent determinations).

Certified reference waters SLRS-3 from the Canadian National Research Council (certified Cr value = 0.3 ± 0.03 μg/L) and 1643d from NIST (18.5 ± 0.2 μg/L) were included in each analysis (n = 9) and measured concentrations were 98 ± 10% of the certified value for SLRS-3 and 101 ± 4% for 1643d.

Although ICP-MS is unable to detect changes in the oxidation state of Cr during our experiments, Cr(III) oxidation in circumneutral solutions and in the absence of suitable oxidants such as Mn oxides and hydroxyl radicals (neither of which is present in ISO medium) is expected to be rather slow (Eary and Rai 1987; Lin 2002). Furthermore, four solutions of Cr(VI) (chromate and dichromate salts) were prepared in ISO medium at concentrations of

Table 1 Temporal variation (72 h) of Cr concentrations in ISO medium solutions amended with either $K_2Cr_2O_7$ or Na_2CrO_4

Time (h)	C1	C2	C3	C4
	$K_2Cr_2O_7$			
0	2487 ± 22	1215 ± 21	751 ± 6.5	160 ± 5.5
24	2489 ± 53	1210 ± 20	751 ± 13	157 ± 12
48	2502 ± 15	1195 ± 27	729 ± 20	147 ± 7.0
72	2493 ± 8.5	1208 ± 31	740 ± 19	147 ± 5.4
	Na_2CrO_4			
0	2519 ± 42	1301 ± 23	789 ± 7.8	161 ± 16
24	2511 ± 43	1308 ± 14	737 ± 24	155 ± 0.60
48	2543 ± 19	1294 ± 18	727 ± 12	144 ± 5.8
72	2545 ± 18	1312 ± 14	743 ± 22	144 ± 7.9

All values (±1 analytical s.d.; n = 1) are in μg/L of Cr. Solutions prepared in non acid-washed microplates. No statistics possible

Table 2 Temporal variation (72 h) of Cr concentrations in ISO medium solutions amended with either $(Cr(NO_3)_3 \cdot 9H_2O$ or $CrCl_3 \cdot 6H_2O$

Time (h)	C1	C2	C3	C4
	$Cr(NO_3)_3$			
0	2244 ± 16^a	433 ± 4.6^a	90.7 ± 1.02^a	18.0 ± 0.08^a
24	618 ± 165^b	195 ± 3.1^b	37.9 ± 0.31^b	9.3 ± 0.26^b
48	$363 \pm 2.4^{b*}$	183 ± 0.7^c	$26.2 \pm 0.57^{c*}$	$7.8 \pm 0.03^{c*}$
72	$274 \pm 4.7^{b*}$	75 ± 0.9^d	$22.7 \pm 0.36^{c*}$	$7.1 \pm 0.2^{c*}$
	$CrCl_3$			
0	2165 ± 43^a	412 ± 7.5^a	74.1 ± 0.48^a	14.2 ± 0.01^a
24	587 ± 40^b	280 ± 10.8^b	46.5 ± 0.56^b	8.5 ± 0.26^b
48	588 ± 38^b	270 ± 16.9^b	34.6 ± 0.35^c	6.3 ± 0.12^c
72	81 ± 16^c	145 ± 14.2^c	29.6 ± 0.44^d	5.0 ± 0.36^d

All values (mean $\pm$ 1 s.d.; n = 2 for $Cr(NO_3)_3$; n = 3 for $CrCl_3$) are in µg/L of Cr. For $Cr(NO_3)_3$, * indicates those cases where only one measurement ($\pm$1 analytical s.d.) was available. Solutions prepared in non acid-washed microplates. Different superscripts indicate statistically significant differences among sampling times (one-way ANOVA; $p < 0.01$)

Table 3 As in Table 2, but for solutions prepared in acid-washed microplates

Time (h)	C1	C2	C3	C4
	$Cr(NO_3)_3$			
0	2276 ± 10^a	437 ± 25^a	88.9 ± 2.43^a	17.0 ± 1.10^a
24	610 ± 18^b	159 ± 11^b	34.5 ± 1.34^b	6.6 ± 0.29^b
48	185 ± 11^c	65 ± 0.2^c	22.8 ± 0.26^c	6.0 ± 0.01^b
72	90 ± 1^d	28 ± 1.8^c	13.8 ± 0.55^d	5.3 ± 0.24^b
	$CrCl_3$			
0	2699 ± 45^a	436 ± 32^a	85.5 ± 2.89^a	18.1 ± 0.55^a
24	1021 ± 36^b	160 ± 7.2^b	37.6 ± 1.29^b	5.9 ± 0.21^b
48	455 ± 12^c	58 ± 2.7^c	23.9 ± 1.69^c	5.6 ± 0.23^b
72	278 ± 7^d	20 ± 0.4^c	16.5 ± 0.65^d	6.1 ± 0.27^b

All values (mean $\pm$ 1 s.d.; n = 3 for t = 0, 24; n = 2 for t = 48, 72) are in µg/L of Cr. Different superscripts indicate statistically significant differences among sampling times (one-way ANOVA; $p < 0.01$)

2,600; 1,300; 700; and 160 µg/L of Cr. In these solutions, the measured Cr concentrations remained within 10% of expected initial values over 72 h (Table 1). Literature-derived knowledge and the overall temporal stability of Cr(VI) concentrations (as opposed to the behavior of Cr(III)—see Results and discussion) support the hypothesis that substantial Cr(III) oxidation does not occur in ISO medium; at least in the absence of algae. The total Cr measured by ICP-MS was therefore assumed to be in the trivalent form.

Chromium speciation in ISO medium was estimated with the software package VisualMinteq version 2.40b (http://www.lwr.kth.se/english/OurSoftware) using the Cr

concentrations measured at t = 0 as input for the component Cr(III) and the tabulated composition of ISO medium (ISO 2004) as input for the other model components. No changes were made to the default complexation constants contained in the model database. The pH of the chromium solutions was measured at 0, 24, 48, and 72 h. Addition of Cr(III) to ISO medium did not change pH by more than 0.5 units and, for each solution, the mean of the four measured pH values was used for the speciation calculation (see next section).

Results and Discussion

For experiments in ISO medium, measured Cr concentrations at t = 0 were within 20% of the expected values (see Tables 2 and 3 for the 72-h experiments). Small Cr losses may have occurred in the stock solutions (which were not stabilized by the addition of e.g., sulfuric acid), but the discrepancies between expected and observed concentrations at t = 0 do not invalidate the conclusions of this study. Measurements' repeatability was better than 5% and concentrations among replicate experiments agreed to within 5–10%.

In the 72 h-experiments in ISO medium, total chromium concentrations decreased by 60–90% of their initial values over 72 h. The largest decreases had already occurred after 24 h and were more marked in acid-washed microplates (Tables 2 and 3). In some cases (solutions C2 and, chloride salt only, C1 for non acid-washed microplates—Table 2), the decrease in Cr concentration was not monotonic and further research will be required for a full understanding of between-experiments variability. However, all 72 h-experiments confirmed decreasing trend of Cr concentration with time; which is the key point for this study. In the 24 h-experiments in ISO medium (Table 4), Cr concentrations remained within 30% (nitrate salt) and 20% (chloride salt) of their initial values for t ≤ 5 h; while concentrations after 24 h were comparable to those measured in the 72 h-experiments. Finally, in the 72 h-experiments in MilliQ water (Table 5), chromium concentrations remained within 20% of the initial values.

The similar results for experiments in preconditioned and non preconditioned microplates (Tables 2 and 3), the limited Cr losses in ISO medium for t ≤ 5 h (Table 4), and the stability of Cr concentrations in MilliQ water for t = 72 h (Table 5) suggest that no more than 20–30% of Cr can be lost from the solutions by adsorption onto the microplates' walls. Other mechanisms are therefore necessary to explain the observed 60–90% decreases in Cr concentrations during 72 h ISO experiments.

According to model calculations (Table 6), the predominant Cr species in ISO medium would be $Cr(OH)_3(aq)$

Table 4 Temporal variation (24 h) of Cr concentrations in ISO medium solutions amended with either $(Cr(NO_3)_3 \cdot 9H_2O$ or $CrCl_3 \cdot 6H_2O$

Time (h)	C1	C2	C3	C4
	$Cr(NO_3)_3$			
0	2176 ± 54^a	498 ± 8.7^a	87.8 ± 3.62^a	17.6 ± 0.50^a
1	2115 ± 17^a	441 ± 4.0^b	77.6 ± 2.95^b	15.7 ± 0.23^b
3	1789 ± 10^b	392 ± 20.4^c	64.7 ± 1.37^c	14.3 ± 0.11^c
5	1601 ± 25^c	361 ± 32.5^c	57.8 ± 2.57^d	12.5 ± 0.11^d
24	486 ± 23^d	292 ± 13.2^d	33.4 ± 0.50^e	5.1 ± 0.06^e
	$CrCl_3$			
0	2650 ± 78^a	473 ± 8.3^a	91 ± 0.5^a	18.1 ± 0.27^a
1	2379 ± 74^b	430 ± 4.7^b	84 ± 2.0^a	17.2 ± 0.31^b
3	2210 ± 36^c	390 ± 10^c	72 ± 1.3^b	15.0 ± 0.03^c
5	2127 ± 57^c	367 ± 4^d	73 ± 10.2^b	14.2 ± 0.27^d
24	740 ± 128^d	297 ± 8^e	42 ± 1.3^c	8.5 ± 0.13^e

All values (mean $\pm$ 1 s.d.; n = 2 for $Cr(NO_3)_3$; n = 3 for $CrCl_3$) are in µg/L of Cr. Solutions prepared in non acid-washed microplates. Different superscripts indicate statistically significant differences among sampling times (one-way ANOVA; $p < 0.05$)

Table 5 Temporal variation (72 h) of Cr concentrations in MilliQ water amended with either $(Cr(NO_3)_3 \cdot 9H_2O$ or $CrCl_3 \cdot 6H_2O$

Time (h)	C1	C2	C3	C4
	$Cr(NO_3)_3$			
0	2256 ± 26^a	426 ± 1.6^a	94 ± 4.1^a	18.8 ± 0.06^a
72	2251 ± 53^a	424 ± 2.4^a	84 ± 0.4^b	18.6 ± 0.03^b
	$CrCl_3$			
0	2202 ± 84^a	432 ± 1.8^a	70.4 ± 4.8^a	12.3 ± 0.72^a
72	2202 ± 183^a	379 ± 23.4^b	81 ± 3.0^b	9.6 ± 0.25^b

All values (mean $\pm$ 1 s.d.; n = 3) are in µg/L of Cr. Solutions prepared in non acid-washed microplates. Different superscripts indicate statistically significant differences among sampling times (t-test; $p < 0.05$)

for solutions C1 and C2 and chromium-EDTA complexes for solutions C3 and C4 (EDTA is added to ISO medium to prevent precipitation of Fe). Charged chromium forms would account for less than 5% of total concentrations in all cases. Supersaturation with respect to the solid phase $Cr(OH)_3$(am) is predicted only for solutions C1 and C2 of both salts (Table 6). Despite the presence of EDTA, the speciation model also predicts supersaturation for ferrihydrite, but the actual formation of solid phases also depends on kinetic considerations that are not taken into account in thermodynamic speciation models such as VisualMinteq.

The speciation results suggest at least three mechanisms to explain the temporal decrease of chromium levels in ISO medium: precipitation of $Cr(OH)_3$(am), formation of mixed Cr–Fe hydroxides (which are much less soluble than pure Cr hydroxides—Rai et al. 1989—and could precipitate in solutions C3 and C4), and Cr adsorption on freshly formed Fe

hydroxides. The available speciation calculations do not predict the theoretical extension of the second and third process, because the constants for the corresponding solubility and adsorption equilibria are not included in the model. A more detailed study of chromium chemistry, including the determination of the unknown chromium-related constants in ISO medium, would be necessary to understand the relative importance of the different mechanisms.

Various experimental factors (e.g., the use of Erlenmeyer flasks instead of microplates or the use of another culture media such as AAP) could obviously influence the temporal evolution of Cr concentrations. In ancillary experiments performed in Erlenmeyer flasks under agitation (solutions C1, C2, C3, and C4; chloride salt in ISO medium), Cr concentrations decreased by 70–80% in 72 h (Table 7); in good agreement with results for microplates. Chromium concentration in AAP medium (nitrate salt, C1 only) decreased by 85% in 72 h (from 2259 ± 23 µg/L at t = 0 to 393 ± 41 µg/L at t = 72); in good agreement with observations in ISO medium. The tendency of Cr(III) concentrations to decrease markedly with time in buffered algal media therefore seems a general phenomenon; although, in an actual ecotoxicological test, the variations in Cr concentrations would be further modified by the presence of algae.

Such cumulative evidence is important because, with few exceptions (Meisch and Schmitt-Beckmann 1979; Walsh and O'Halloran 1997), inorganic Cr salts have generally been used to compare the toxicity or bioaccumulation of Cr(III) and Cr(VI) in algae and other organisms. For trivalent chromium, many EC50s reported for algae (but also other organisms; see references in INERIS 2005; Munn et al. 2005; Pawlisz et al. 1997) fall in the range for which saturation of the test solutions with respect to $Cr(OH)_3$(am) can be expected. The results of this work show that large decreases in chromium concentrations can also occur in unsaturated solutions for reasons other than adsorption onto containers' walls. The ecotoxicology of Cr(III), and its relative toxicity with respect to Cr(VI), may therefore require a re-assessment using ecotoxicological models capable of accounting for the progressive loss of toxicant from the test medium or flow-through experiments to maintain constant Cr concentrations during the tests. In the former case, experimental verification of the temporal variability of chromium concentration in the test solutions (and in a solution of concentration corresponding to the calculated, nominal EC50 value) will obviously be necessary.

Even if the intrinsic toxicity of Cr(III) turns out to be much higher than previously thought, its bioavailability in field conditions may still remain low. Chromium (III) is usually rapidly removed from the water column and supersaturation with respect to chromium solid phases is

Table 6 Measured pH values and results of speciation calculations for the various Cr solutions in ISO medium

Solution	pH	Charged species[a]	EDTA complexes[b]	$Cr(OH)_3(aq)$	Supersaturation predicted[c]
	$Cr(NO_3)_3$				
C1	7.7 ± 0.2	4.6	4.5	90.9	Yes
C2	8.1 ± 0.1	1.6	21.1	77.3	Yes
C3	8.2 ± 0.1	0.5	66.4	33.1	No
C4	8.2 ± 0.1	0.2	87.1	12.7	No
	$CrCl_3$				
C1	8.1 ± 0.1	1.9	3.0	95.1	Yes
C2	8.2 ± 0.1	1.4	14.3	84.4	Yes
C3	8.4 ± 0.1	0.5	50.6	48.9	No[d]
C4	8.3 ± 0.1	0.2	80.5	19.2	No

Speciation results are given as percent abundance of total Cr concentrations. The measured chromium concentrations at t = 0 (Table 1) were used as input values. pH values (± 1 s.d.) are averages of four independent measurements taken at 0, 24, 48, and 72 h. See text for more details

[a] $Cr(OH)_2^+$, $Cr(OH)^{2+}$, $Cr(OH)^{4-}$

[b] $CrEDTA^-$, $CrOHEDTA^{2-}$

[c] With respect to $Cr(OH)_3(am)$

[d] Saturation limit

Table 7 Temporal variation (72 h) of Cr concentrations in ISO medium solutions amended with $CrCl_3 \cdot 6H_2O$ in Erlenmeyer flasks

Time (h)	C1	C2	C3	C4
	$CrCl_3$			
0	2381 ± 24^a	467 ± 3.1^a	86.7 ± 2.63	18.0 ± 0.52
24	1123 ± 102^b	259 ± 10.5^b	47.3 ± 0.72	11.0 ± 0.27
48	$864 \pm 212^{b,c}$	159 ± 6.6^c	37.2 ± 0.72	6.11 ± 0.15
72	614 ± 177^c	99 ± 21.2^d	28.5 ± 2.40	4.92 ± 0.10

All values are in µg/L of Cr. Errors are given as ± 1 standard deviation on three experiments for C1 and C2 and as analytical error on one measurement for C3 and C4. No results available for $Cr(NO_3)_3 \cdot 9H_2O$. Different superscripts indicate statistically significant differences among sampling times (one-way ANOVA; $p < 0.05$). No statistics possible for C3 and C4

expected only in zones directly impacted by, e.g., tannery wastewaters (Koukal et al. 2004). Furthermore, the influence of ligands such as sulfides and organic matter, which are absent in ISO medium but occur in natural environments (Rozan et al. 2000; Town and Filella 2002), should also be considered. In any case, given the plans to replace Cr(VI) by Cr(III) in electroplating processes (Baral et al. 2006), more detailed studies on the possible chronic effects of Cr(III) in standard media and/or environmental matrices at concentrations not exceeding its solubility probably deserve some priority in future research and risk assessment.

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