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Chromium(VI) is more toxic than chromium(III) to freshwater algae: A paradigm to revise?

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ABSTRACT

The behavior and toxicity of Cr(III) and Cr(VI) to the green algae *Pseudokirchneriella subcapitata* and *Chlorella kessleri* were studied in a standard culture medium (ISO medium) and, for *P. subcapitata* only, in ultrafiltered natural water enriched with all ISO components (modified ISO medium). In all solutions amended with Cr(III), initial chromium concentrations decreased by 60–90% over 72 h (the duration of algal tests) indicating that protocols for testing poorly soluble substances are required to properly evaluate Cr(III) toxicity. After accounting for its behavior in test solutions, chromium(III) was 5–10 times more toxic than Cr(VI) in both media. For *P. subcapitata*, the average 72 h EC50 of Cr(III) in ISO medium was 17.4 ± 4.7 μg/L (n=9); lower than corresponding hardness-corrected Continuous Concentration Criteria of the US EPA and well within the range of Cr concentrations found in waters impacted by tannery discharges. These results follow from intrinsic chemical properties of Cr(III) in circumneutral solutions, so that the actual toxicity of Cr(III) to aquatic organisms may be generally underestimated.

1. Introduction

The importance of speciation in controlling the environmental fate, toxicity and bioavailability of trace elements is now well established. In the case of chromium, a widely-used element in industry (Stoecker, 2004), specific attention has been devoted to establish the relative toxicity to aquatic organisms of trivalent and hexavalent redox forms; i.e., the predominant oxidation states in surface waters. Most of the published literature (Pawlisz et al., 1997; Stoecker, 2004; Munn et al., 2005; Shanker et al., 2005 and the US EPA Ecotox database — http://cfpub.epa.gov/ecotox) concludes that hexavalent Cr is more toxic/bioavailable than trivalent Cr; although data in a few recent studies do not conform to this conclusion (Thompson et al., 2002; Kaszycki et al., 2004; Ksheminska et al., 2005; Pereira et al., 2005).

In a recent work (Vignati et al., 2008), we showed that Cr(III) concentrations added to standard algal test media decreased by 60–90% over 72 h (the typical duration of algal toxicity tests); while Cr(VI) concentrations remained within $\pm 20\%$ of the initial concentrations during an equal span of time. The need for experimentally verifying exposure concentrations during toxicity tests has long been recognized and, in the case of poorly soluble substances, specific recommendations have been proposed by authoritative, international bodies (OECD, 2000; ISO, 2006). However, with the exception of dichromium oxide, Cr(III) inorganic salts used in toxicity tests are rather soluble (Table S1) and hence not covered by these (or analogous) guidelines. The issue with Cr(III) inorganic salts is that they undergo hydrolysis and form Cr hydroxides at circumneutral pH (Sass and Rai, 1987; Rai et al., 1989), which includes the typical pH values of standard algal culture media (e.g., ISO, pH=8.3 and AAP, pH=7.5). Formation of hydroxides can reduce the solubility of Cr(III) to below 5 μg/L (Sass and Rai, 1987; Rai et al., 1989); much lower than effect concentrations (ECs50) and No-Observable Effect Concentrations (NOECs) reported in several review documents (Pawlisz, 1997; INERIS, 2005; Munn et al., 2005). This solubility limit is also below the criterion maximum concentration (CMC) and the criterion continuous concentration (CCC) of 574 and 74 μg/L proposed by US EPA (2006) and very close to the Predicted No Effect Concentration (PNEC) of 4.7 μg/L established by the European Union assessment report on chromium compounds (Munn et al., 2005).

Given all the above considerations, it is particularly noteworthy that in early studies on algae (Den Dooren de Jong, 1965; Meisch and Schmitt-Beckmann, 1979; Turbak et al., 1986; Greene

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et al., 1988; IUCLID, 1999) subsequently used for deriving Canadian and European guidelines on Cr(III) (Pawlisz et al., 1997; INERIS, 2005; Munn et al., 2005) the actual Cr(III) concentrations in the exposure media were either not measured or measured only at the beginning of the experiment. Because the concentrations of Cr in test solutions amended with Cr(III) can markedly decrease with time (see previous paragraph and Vignati et al., 2008), it is therefore possible that the toxicity of Cr(III) to freshwater algae is strongly underestimated.

In this study, the temporal variability of trivalent and hexavalent chromium concentrations and the relative toxicity of the two Cr redox forms to the algae *Pseudokirchneriella subcapitata* and *Chlorella kessleri* were studied in a standard culture medium (ISO medium) and, for *P. subcapitata* only, in modified ISO medium consisting in ultrafiltered natural water enriched with all the components of standard ISO. The aim of this work was to examine how appropriate consideration of Cr(III) chemistry could affect the interpretation of the relative toxicity of trivalent and hexavalent chromium to freshwater algae and to verify if the current paradigm "Cr(VI) is more toxic than Cr(III)" required some revision.

2. Materials and methods

2.1. Temporal stability of chromium concentrations and quality control procedures

Stock solutions of Cr(III) (2.38 g/L) and Cr(VI) (2.6 g/L) were prepared in ultrapure water using analytical grade $Cr(NO_3)_3 \cdot 9H_2O$ (VWR, purity >98%) for trivalent chromium and $K_2Cr_2O_7$ and Na_2CrO_4 (both from VWR, purity >99.5%) for hexavalent chromium. Four working solutions of each salt (see Table 1) were prepared as needed in standard ISO medium 8692 (hereinafter ISO medium) (ISO, 2004), in ultrafiltered Lake Geneva water or in modified ISO medium (see below) immediately prior to use. The concentration range of the working solution covered the range of ECs50 and NOECs reported in the literature for algae. The Cr nitrate and dichromate solutions were analyzed for possible contamination by Cu, Ni, Pb, Sb, and Zn, whose concentrations were below detection limits (Cu, Pb, Sb) or less than 1 μg/L (Ni, Zn).

Table 1
Temporal variability of Cr concentrations in standard ISO medium, ultrafiltered Lake Geneva water (UF water), and modified ISO medium amended with $Cr(NO_3)_3 \cdot 9H_2O$ and, for UF water only, $K_2Cr_2O_7$ or Na_2CrO_4 (see text for details).

Time (h)	C1	C2	C3	C4
$Cr(NO_3)_3$ (standard ISO)[$]				
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}	$22.7 \pm 0.36^{c*}$	$7.1 \pm 0.2^{c*}$
$Cr(NO_3)_3$ (UF Water)				
0	2147 ± 30^{a}	433 ± 6.2^{a}	87.9 ± 0.21^{a}	18.8 ± 0.06^{a}
24	105 ± 33^{b}	87.2 ± 8.8^{b}	26.8 ± 0.67^{b}	8.6 ± 0.04^{b}
48	40 ± 10^{b}	29.8 ± 3.2^{b}	21.3 ± 0.18^{b}	$7.0 \pm 0.60^{b,c}$
72	59 ± 19^{b}	28.2 ± 21.2^{b}	18.6 ± 3.12^{b}	5.5 ± 0.08^{c}
$Cr(NO_3)_3$ (modified ISO)				
0	2127 ± 15^{a}	432 ± 0.7^{a}	83.1 ± 0.51^{a}	17.5 ± 0.01^{a}
24	90 ± 11^{b}	160 ± 6.6^{b}	30.3 ± 1.37^{b}	6.6 ± 0.21^{b}
48	28 ± 2.2^{c}	43.1 ± 0.6^{c}	19.8 ± 1.58^{c}	3.5 ± 0.26^{c}
72	15 ± 0.7^{c}	19.3 ± 1.7^{d}	8.1 ± 0.67^{d}	1.7 ± 0.05^{d}
$K_2Cr_2O_7$ (UF water)				
0	2497 ± 12^{a}	1283 ± 5.1^{a}	785 ± 26^{a}	140 ± 0.2^{a}
24	$2574 \pm 1^{a,b}$	1305 ± 2.2^{a}	800 ± 8^{a}	142 ± 0.4^{a}
48	$2649 \pm 31^{b,c}$	1336 ± 21^{a}	825 ± 19^{a}	152 ± 0.6^{b}
72	2695 ± 8^{c}	1365 ± 0.9^{b}	835 ± 82^{a}	154 ± 2.2^{b}
Na_2CrO_4 (UF water)				
0	2532 ± 58^{a}	1371 ± 6.2^{a}	796 ± 20^{a}	155 ± 0.7^{a}
24	2591 ± 14^{a}	1332 ± 15^{a}	810 ± 4.9^{a}	156 ± 0.5^{a}
48	2666 ± 11^{a}	1367 ± 13^{a}	847 ± 2.7^{a}	161 ± 1.1^{b}
72	2659 ± 45^{a}	1336 ± 28^{a}	855 ± 5.3^{a}	164 ± 0.6^{b}

Expected concentrations (from C1 to C4): 2380; 476; 95.2; and 19 μg/L for $Cr(NO_3)_3 \cdot 9H_2O$ and 2600; 1300; 770; and 160 μg/L for $K_2Cr_2O_7$ and Na_2CrO_4. All values (mean ± 1 s.d.; $n=2$ or '*' $n=1 \pm 1$ analytical s.d.) are in μg/L of Cr. Different superscripts indicate statistically significant differences among sampling times (one-way ANOVA; $p<0.01$).

[$] Data from (Vignati et al., 2008).

Modified ISO medium (mISO) was prepared using ultrafiltered water from Lake Geneva, instead of ultrapure water, as a dilution matrix for the various components of ISO medium. Lake Geneva has the following typical composition (values in mg/L unless otherwise specified): Ca=45; Mg=6; Na=5.6; K=1.4; Cl=8.1; SO_4=47.2; silica=1.9; total N=0.68; total P=0.027; pH about 7.9 after winter mixing, conductivity=302 μS/cm, total hardness=2.8 meq/L (140 mg/L $CaCO_3$) and alkalinity of 1.8 meq/L (Strawczynski, 2006). Organic carbon and total Cr concentrations were directly measured in the ultrafiltered water and were about 1.4 mg/L and 0.4 μg/L respectively. Ultrafiltration of Lake Geneva water was performed using a regenerated cellulose membrane (Prepscale, Millipore; nominal cut-off 300 kDa) according to Kottelat et al. (2008). The use of ultrafiltered water was selected to reduce the amount of colloids present in mISO because colloids have a major influence on metal toxicity (Koukal et al., 2003, 2007). The experiments with mISO were started on the day following the completion of ultrafiltration. Reconstituting the complete ISO medium in natural water rather than simply adding the nitrogen and phosphorus salts allowed comparison of the matrix effects between ISO and mISO, while eliminating possible confounding effects due to differences in micronutrient availability to algae and to the presence of EDTA. Note that no algal growth was observed in UF water without N and P addition after 72 h.

Aliquots of each working solution were transferred into sterilized polystyrene 96-well microplates (300 μL per well) and then recovered for Cr analysis after 0, 24, 48, and 72 h. Note that only 200 μL of solution were recovered from each well in order to avoid resuspending and sampling Cr precipitates possibly deposited on the bottom of the wells. Freshly formed colloidal particles could have been sampled in this way, but we chose not to filter the samples to provide a more realistic estimate of the Cr concentration to which algae should be exposed during the test. Between recoveries, microplates were kept under the same conditions used for the algal toxicity tests (see next section). The details of the entire procedure for these abiotic experiences have been previously described (Vignati et al., 2008).

In all experiments, total chromium concentrations were determined by ICP-MS (Agilent, HP4500) at m/z 53 using external calibration (range 0–200 μg/L) and internal standard correction (^{103}Rh). Depending on concentration, chromium solutions were diluted between 6- and 101-fold to avoid excessive matrix interference and to keep the Cr levels within the calibration range. For solutions prepared in standard ISO, the ICP-MS figures of merit and the analytical quality control procedures were reported in Vignati et al. (2008). For solutions prepared in mISO, the detection limit (mean ± 1 standard deviation) was 0.1 ± 0.09 μg/L for 3 independent series of analyzes. Accuracy was not verified in these analytical runs, but the correspondence between expected and measured values at the beginning of the experiments (Table 1) testified a satisfactory performance. Although ICP-MS is unable to detect changes in Cr redox state, we already provided several reasons to regard as highly unlikely changes in the oxidation state of Cr in abiotic ISO medium solutions (Vignati et al., 2008). The reported analytical Cr concentrations can therefore be assumed to provide a reasonable estimate of Cr(III) or Cr(VI) present in the various solutions.

2.2. Chromium toxicity

Immediately prior to the tests, chromium stock solutions were diluted 1000-fold to 2.38 mg/L for Cr(III) and 2.6 mg/L for Cr(VI). Eight sequential dilutions were then prepared in ultrapure water (or in ultrafiltered Lake Geneva water) following a geometric range with a separation factor of 2; giving a total of nine Cr concentrations for algal exposure. Aliquots (270 μL) of each dilution (ultrapure water for controls) were micropipetted into 96-well microplates (8 rows by 12 columns). External wells (rows 1 and 8; columns 1 and 12) were filled with 300 μL of ultrapure water to minimize evaporation. The remaining 60 wells were filled as described earlier (Ferrari et al., 2006a, 2006b): columns 3–11 (6 wells per column) received the 270 μL of increasing Cr dilutions; while control wells (column 2) received 270 μL of ultrapure water.

Preparation of the algal inoculum for the toxicity tests was carried out according to the ISO 8692 guideline (ISO, 2004). An adequate amount of ISO or mISO (at a concentration 10 times the one necessary to ensure algal growth) was prepared using sterilized, concentrated nutrient solutions. Cells of the alga *P. subcapitata* (ATCC 22662, Rockville, MD) in exponential growth phase were added to the concentrated ISO or mISO at a density of about 200,000 cells/mL. The exact number of cells was enumerated using a Colter Counter (model ZM, 70-μm cell aperture, Colter Electronics, Toronto, Ont., Canada). The algal culture was maintained in oligo-LC medium (Ferrari and Férard, 1996) and transferred without washing.

Thirty microliters of algal inoculum in 10-fold concentrated ISO medium were then added to the wells containing the 270 μL of Cr solutions and to the control