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Article

Reductive Transformation of Hexavalent Chromium in Ice Decreases Chromium Toxicity in Aquatic Animals

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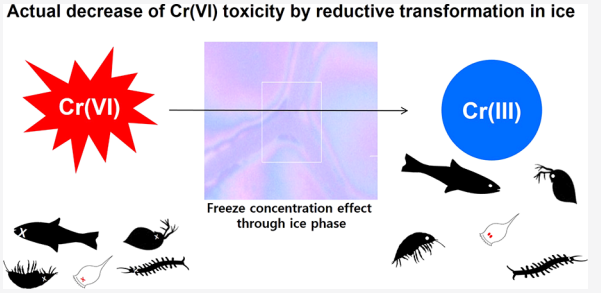
ABSTRACT: In this study, the toxicity of hexavalent chromium [Cr(VI)] reduced by citric acid in ice was measured using representative aquatic model invertebrates (*i.e.*, rotifer, water flea, amphipod, and polychaete) and a vertebrate (zebrafish) by analyzing short- and/or long-term endpoints that are frequently applied to each animal. Cr(VI) reduction in the presence of citric acid was markedly enhanced in the ice phase compared to that in an aqueous solution through the freeze concentration effect. The highly concentrated Cr(VI) and citric acid in ice grain boundaries were also confirmed using *in situ* cryogenic confocal Raman spectroscopy. Overall, exposure to Cr(VI) resulted in higher acute and/or chronic effects on aquatic animals, such as drastic mortality, growth inhibition, and decrease in offspring number, whereas the animals were increasingly tolerant to Cr(VI) that was reduced in the ice phase. Sublethal concentrations of Cr(VI) significantly decreased the antioxidant capacity in the aquatic animals. However, when the same concentrations of Cr(VI) were reduced in ice, these treatments showed no modulation or increase in the antioxidant defense system. Taken together, our results suggest that Cr(VI) reduction into Cr(III) was successfully achieved in ice and that this methodology can decrease the actual toxicity of Cr(VI) in aquatic animals.



KEYWORDS: *chromium toxicity, chromium reduction, aquatic animal, toxicity endpoint, chemical reaction in ice*

1. INTRODUCTION

Chromium (Cr), a transition metal, is considered a toxic element that exists in two main stable redox forms in surface waters, namely trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)].[1] Industrial processes and anthropogenic activities have been suggested as the main source of Cr pollution through release from the production of refractory materials, metallurgy, cement-producing plants, chemical manufacturing, and wood preservation to environments.[2] Groundwater in ultramafic bedrock has also been recognized as a major source of Cr contamination.[3,4] Hexavalent Cr is highly mobile, soluble, and highlighted to be of ecotoxicological concern compared to the relatively immobile, poorly soluble, and less or nontoxic Cr(III).[5] These characteristics of Cr(VI) lead to its wide dispersion through pollution of both surface and underground water resources; aquatic contamination has thus become a major global environmental concern involving detection in drinking water and subsequent accumulation in human and animal bodies.[6] Environmental concentrations of Cr in aquatic environments have been reported to be 2−50 (sometimes more), 0−84, and 0.2−1 μg L^{-1} of Cr in groundwater, surface water, and rainwater, respectively.[4,7] The mean Cr concentration was suggested to be 0.3 μg L^{-1} in oceans.[8]

Public concerns are primarily related to Cr(VI) as being the most toxic element among the redox forms of Cr in aquatic environments due to its ability to directly penetrate biological membranes, stability and persistence in general aquatic environments, high water solubility, and oxidation potential.[9] Mechanisms of Cr(VI) toxicity, tolerance, and detoxification have been suggested in prokaryotes and eukaryotes. In microorganisms, Cr(VI) is transported into cells through oxyanion transporters due to structural similarity between Cr(VI) and phosphate or sulfate anions.[10] Hexavalent Cr is classified as genotoxic and carcinogenic to humans and animals.[11−13] Among aquatic animals, the majority of studies on Cr(VI) toxicity (*e.g.*, immunotoxicity, developmental toxicity, and reproductive toxicity) to date have focused on fish.[14−16] In several studies, Cr(VI) has been commonly shown to cause acute/chronic toxicity and physiological damage to aquatic invertebrates at individual and/or population levels, causing oxidative stress, tissue damage, neurotransmission dysfunction, diminished energy metabolism, reduced fitness, retarded growth, and/or mortality.[17−22] However, most studies on Cr(III) showed no observable or insignificant effects on

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aquatic animals at environmentally relevant and even high concentrations.

Natural reduction of Cr(VI) to Cr(III) occurs through chemical biological processes and is favorable in acidic environments following the reaction: $HCrO_4^- + 7H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O$. However, this reaction occurs extremely slowly in the solution phase.[23] From recent studies, metal oxides such as iron oxides or manganese oxides showed a pronounced catalytic effect at low pH on the soil surface.[24] They also strongly affect the reduction of Cr(VI) by organic compounds such as oxalic acid, α-carbonyl carboxylic acids, α-hydroxyl carboxylic acids, and substituted phenols.[25] Citric acid, which is abundant in nature, especially in the metabolism of all aerobic organisms, is also an effective organic reductant for Cr(VI) reduction in nature.[26] Most chemical reactions occur slowly with decreasing temperature; in contrast, several chemical reactions such as reductive dissolution of metal oxides,[27,28] iodine activation from iodate (IO_3^-),[29] and transformation of bromate (BrO_3^-) to organobromine compounds in the presence of humic substances[30] are accelerated in ice media than in aqueous solutions. These phenomena that seem to disobey the Arrhenius equation occur because of the "freeze concentration phenomenon" during the freezing process. Frozen solutions contain a small quantity of liquid between bulk ice crystals, which is generally referred to as the ice grain boundary or liquid brine. Freeze concentration indicates the strong concentration effect of solutes and protons (in acidic conditions) in the solution of ice grain boundaries by exclusion from bulk ice crystals during the freezing process.[31] By this phenomenon, chemical reactions occur more rapidly within ice media than in water. Recently, reduction of Cr(VI) by organic compounds in ice[32] was investigated to better understand Cr(VI) reduction in cold areas, such as polar regions, high-latitude areas, and mid-latitude areas in winter. The reduction of Cr(VI) by hydrogen peroxide,[33] nitrite,[34] and ferrous ions[35] is reported to be enhanced when these aqueous solutions are frozen.

Although evidence has accumulated on the decrease of Cr(VI) toxicity through reduction into Cr(III), only limited information is available on actual toxicity changes of the reduction and definite potential effects in aquatic animals. Aquatic model animals ranging from invertebrates (*i.e.*, rotifers, water fleas, amphipods, and polychaetes) to zebrafish were chosen to understand the potential effects of reductive transformed Cr(VI) and to allow comparison of the well-documented Cr toxicity values in each animal. Analysis of acute/chronic toxicity, *in vivo* parameters, and the biochemical responses of the antioxidant defense system are promising biomarkers of metal pollution in aquatic animals. Thus, in this study, these parameters were selected to understand the potentially hazardous effects of Cr(VI) after its reduction into Cr(III) by citric acid on the ice phase. We set the exposure concentration of Cr(VI) based on its toxic ranges established in each model animal. Utilizing this archival collection, we aimed to provide insights regarding the actually decreased toxicity of Cr(VI) through ice-based reduction as a useful method for detoxification platforms.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Detailed descriptions for all chemicals used have been incorporated in the Supporting Information.

2.2. Chemical Analysis. Cr(VI) concentrations were measured by a colorimetric method using 1,5-diphenylcarbazide (DPC).[36] DPC solution was prepared by adding DPC (0.01 g) to a mixture of acetone (5 mL) and sulfuric acid (0.05 mL). For measurement, 0.1 mL of DPC reagent solution was mixed with diluted sample solutions and kept in the dark for an hour. The dilution ratio of sample solutions was 19.25 to make their concentrations in a dynamic range of DPC (generally < 200 μM). The mixed samples were transferred to a 3 mL quartz cuvette, and absorbance was measured at 540 nm using a UV/visible spectrophotometer (Cary 4000, Agilent). A calibration curve of Cr(VI) ($r^2 = 0.9933$) was used to convert the measured absorbance to Cr(VI) concentration. The variation in the measured absorbance within 1 h was less than 5%. As a preliminary test, it was confirmed that the lowered concentration of Cr(VI) was maintained for 3 months in a range of salinity (0−36 PSU) and temperature (10−25 °C), which covers the environmental conditions employed in toxicity experiments.

Detailed descriptions for *in situ* Raman measurement and chemical mapping of frozen samples have been incorporated in the Supporting Information.

2.3. Animal Toxicity Test. Detailed descriptions for all toxicity methods for each animal have been incorporated in the Supporting Information as followed in the standard methodology or our previous studies.

2.4. Measurement of Oxidative Stress Parameters and the Enzymatic Activity of Acetylcholinesterase and Antioxidant Defense System. Detailed descriptions for all materials and methods are incorporated in the Supporting Information as followed in the general methodology.

2.5. Statistical Analysis. Determination of acute toxicity data (*e.g.*, NOEC and LC_{50}) and the corresponding 95% CIs was based on Probit analysis using ToxRat Professional 2.10.3.1 (ToxRat Solutions GmbH, Alsdorf, Germany). All data were presented as the mean ± standard deviation (S.D.) values. Statistical significance was analyzed using the statistics software package, SPSS (ver. 17.0, SPSS Inc., Chicago IL, USA). Significant differences in the variables measured among treatments were tested using one-way analysis of variance (ANOVA). A *post hoc* Tukey HSD test was performed to determine pairwise differences with time and concentration. A type I error probability of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Cr(VI) Reduction in Ice. The reduction of Cr(VI) in ice was quantified using the DPC colorimetric method and was compared with the same experimental condition in an aqueous phase at room temperature. The experiment was conducted at a high chromium concentration ([Cr(VI)] = 3.85 mM), and quantification was carried out by diluting samples with the maximum concentration to 200 μM for detection using the DPC method. The ratio of [Cr(VI)] to [citric acid] was 1:5. In our preliminary experiment, the threshold of reduction ability of citric acid in the solution and ice phase was measured with a range of citric acid ratios from 1:0.5 to 1:5. Although the initial ratio definitely affected Cr(VI) reduction at time zero in both solution and ice, no further Cr(VI) reduction occurred over time, even at high concentrations of citric acid in solution (Figure S1, Supporting Information). The result indicated that Cr(VI) reduction with the citric acid phase was markedly accelerated in ice whereas, in the aqueous solution, it was not

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Environ. Sci. Technol. 2022, 56, 3503−3513

significant (Figure 1A,B). Furthermore, Cr(VI) reduction in the absence of citric acid as an electron donor was negligible

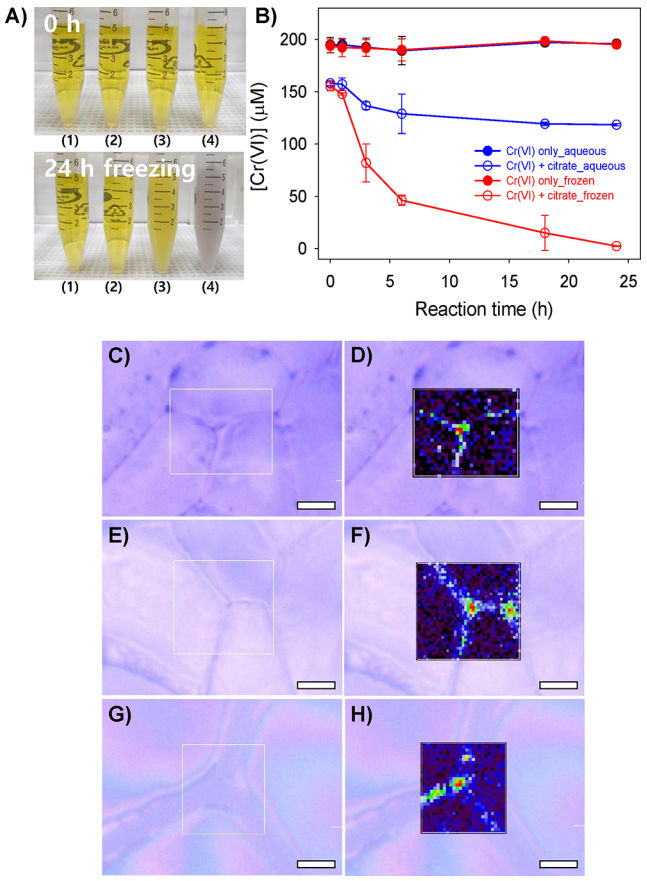

Figure 1. Results of Cr(VI) reduction in water and ice. (A) Visible images of the Cr(VI) reduction experiment (1) Cr(VI) solution (2) mixture of Cr(VI) and citric acid solutions (3) Cr(VI) frozen solution (4) Cr(VI) and citric acid frozen solution (0 h and after 24 h). Experimental conditions: Cr(VI): 3.85 mM, pH 3, [Cr(VI)]/[citric acid] = 1:5. (B) Time profile of Cr(VI) reduction. The initial concentration of Cr(VI) was adjusted to 200 μM. The time point was set to time zero, at which the Cr(VI) solution and citric acid were placed in the low-temperature ethanol bath immediately after mixing. (C−H) optical images and *in situ* Raman chemical mapping images of Cr(VI) (C,D), citric acid (E,F), Cr(VI), and citric acid (G,H) in ice grain boundaries. The Raman intensity was visualized on a rainbow scale (high: red → low: black).

both in ice and solution. The concentration of Cr(VI) in water slowly decreased with reaction time, but the reduction was not completed after 24 h even in the presence of citric acid. However, the reaction proceeded rapidly in ice, and approximately 99.1% of Cr(VI) had reduced into Cr(III) after 24 h (Figure S2, Supporting Information). Citric acid acted as a reducing agent (sodium citrate (E_0 = −0.180 V), which accelerated the reaction. The concentrations of solutes, reducing agents, and protons are reported to be significantly increased in ice grain boundaries between bulk ice crystals upon freezing.[37] During the freezing process, the existing solutes in the solution are excluded from the bulk ice crystals (solid phase) and are confined within the ice grain boundaries (quasi-liquid phase).

To verify the freeze concentration effect, confinement of Cr(VI) and citric acid in the ice grain boundaries were visualized using *in situ* confocal Raman spectroscopy (Figure 1C−H). The concentration of Cr(VI) and citric acid in the ice grain boundaries was shown by a chemical mapping method. The chemical mapping of Cr(VI) and citric acid was represented by the signal intensity in a rainbow color scale (from the strongest to lowest signal, strong: red → weak: black) based on the intensity of the Raman signal for the Cr−O stretching mode (852 cm^{-1}),[38] the dichromate and C−O vibration mode (950 cm^{-1}), andcitric acid,[39] respectively. Each of the Raman signals for Cr(VI) or citric acid could be characterized in the ice grain boundaries when they were frozen separately (Figure 1D,F). The enhanced concentration of Cr(VI) between ice crystals in grain boundaries was observed using *in situ* Raman microscope (Figure S3, Supporting Information). However, the Cr−O peak was almost absent and was undetectable when the two substances (*i.e.*, Cr(VI) and citric acid) were frozen together even when it was measured immediately after freezing (up to 30 min) (Figure 1H). This suggests that rapid redox transformation between Cr(VI) and citric acid occurred simultaneously within the ice grain boundaries.

3.2. Acute Toxicity Test in Rotifers. Exposure to Cr(VI) dose-dependently decreased the survival rate of both rotifers, *Brachionus calyciflorus* (Figure 2A) and *Brachionus plicatilis* (Figure 2B). Acute toxicity tests revealed that Cr(VI) is highly toxic to both species. The 24 h LC_{50} values for Cr(VI) were calculated as 0.3 mg L^{-1} (95% CI 0.19−0.43 mg L^{-1}) for *B. calyciflorus* and 2 mg L^{-1} (95% CI 0.11−0.36 mg L^{-1}) for *B. plicatilis*. For reference, the 24 h LC_{50} values for Cr(III) were calculated as 11.9 mg L^{-1} (95% CI 8.14−14.96 mg L^{-1}) for *B. calyciflorus* and 18.2 mg L^{-1} (95% CI 14.29−24.25 mg L^{-1}) for *B. plicatilis* (Figure S4, Supporting Information). In both species, higher survival rates were observed in the groups treated with Cr(VI) reduced by citric acid in ice compared to those treated with Cr(VI) reduced in water. Equivalent concentrations of citric acid showed no significant mortality in both species.

3.3. Acute Toxicity and Chronic Reproductive Effect in Daphnia. In the acute toxicity test for daphnia, no mortality was observed in the control group for 48 h. LC_{50} values for Cr(VI) were measured as 115 (95% CI 87−143 μg L^{-1}) and 81 μg L^{-1} (95% CI 66−113 μg L^{-1}) at 24 and 48 h, respectively (Figure 3A). Significantly lowered numbers of live offspring per exposed female were observed in a dose-dependent manner compared to the control group during the 21 day test period (P < 0.05) (Figure 3B), though the exposure concentrations ranged within the NOEC values. In the case of Cr(III), the LC_{50} values were calculated to be 1140 (95% CI 789−1314 μg L^{-1}) and 946 μg L^{-1} (95% CI 691−1288 μg L^{-1}) at 24 and 48 h, respectively (Figure S5A, Supporting Information). A dose-dependent effect of Cr(III) concentration was observed on the cumulative number of live offspring per surviving female, with greater effects from 100 μg L^{-1} (P < 0.05), whereas no significant difference was observed at 50 μg L^{-1} (P > 0.05) (Figure S5B, Supporting Information). When the daphnids were exposed to the Cr(VI) that was reduced by citric acid in water (Figure 3C) and ice (Figure 3E), the toxicity values were decreased at both 24 and 48 h, but drastic detoxification was observed in daphnids exposed to Cr(VI) reduced by citric acid in ice than in water. Similarly, more numbers of live offspring were measured in females exposed to Cr(VI) reduced by citric acid in ice (Figure 3D) compared to that in water (Figure 3F).

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Environ. Sci. Technol. 2022, 56, 3503−3513

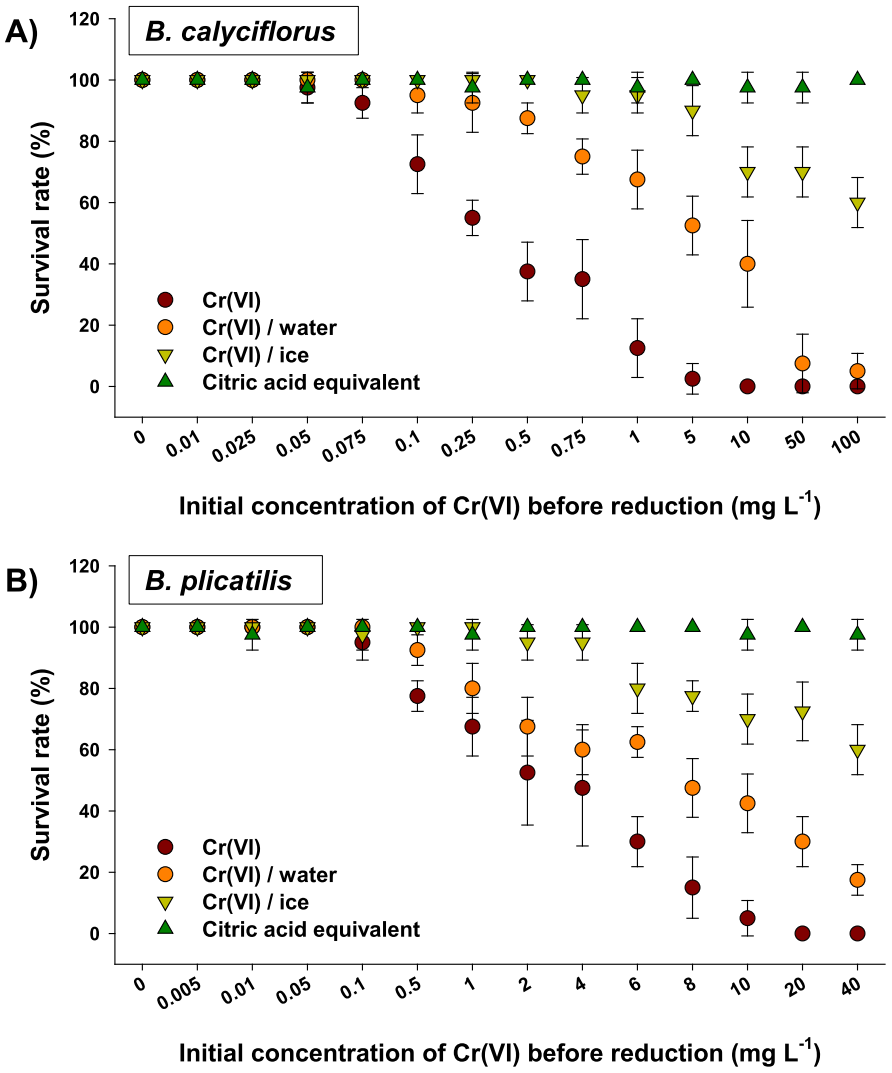

Figure 2. Results of acute toxicity in rotifers. Measurement of 24 h survival rate in (A) *B. calyciflorus* and (B) *B. plicatilis* in response to different concentrations of Cr(VI) (0−100 mg L^{-1} for *B. calyciflorus*; 0−40 mg L^{-1} for *B. plicatilis*), Cr(VI) after reduction in water, Cr(VI) after reduction in ice, and the citric acid equivalent as a reference solvent control. Data are presented as the mean ± S.D. of four replicates (n = 10 per replicate).

3.4. Chronic Toxicity Test in an Amphipod. Exposure to Cr(VI) dose-dependently decreased the *Hyalella azteca* survival rate (Table S1, Supporting Information). The survival rate was significantly lowered by Cr(VI) over 25 μg L^{-1} on day 28 and 6.25 μg L^{-1} on day 42 ($P < 0.05$). Significantly higher toxicity was observed on day 28 in response to 12.5 μg L^{-1} of Cr(VI) reductively transformed in water compared to the group exposed to Cr(VI) only ($P < 0.05$), whereas the overall survival rate was elevated on day 42. Significantly lowered toxicity values were detected in the groups exposed to Cr(VI) reduced by citric acid in ice, and significant differences in survival rate were observed only over 25 μg L^{-1} at days 28 and 42 ($P < 0.05$). In the case of Cr(III), significant decreases in the survival rate were detected at 50 μg L^{-1} on day 28 and over 25 μg L^{-1} on day 42 ($P < 0.05$). However, Cr(VI) toxicity was greater than that of Cr(III), as the survival rates in 50 μg L^{-1} Cr(VI)-exposed amphipods (43% on day 28 and 30% on day 42) were nearly half of the values measured in the 50 μg L^{-1} Cr(III)-exposed group (73% on day 28 and 67% on day 42). The growth rate was not significantly modulated by any treatment ($P > 0.05$).

3.5. Polychaete Toxicity Test. The 96 h LC_{50} value was calculated as 2.59 mg L^{-1} (95% CI 1.42−4.24 mg L^{-1}) for the Cr(VI)-exposed polychaete (Figure 4A). Overall, the toxicity of Cr(VI) reduced by citric acid in ice to the marine polychaete was much lower than that of Cr(VI) alone and Cr(VI) reduced in water. The burrowing ability of the marine polychaete was dose-dependently delayed by Cr(VI) exposure (Figure 4B). The polychaete quickly disappeared into sand within 8 min upon both control and citric acid equivalent exposure, whereas the burrowing ability was more delayed in the 96 h LC_{50}-exposed polychaete compared to that upon exposure to Cr(VI) reduced by citric acid in ice (Figure 4B).

3.6. Zebrafish Early Life Stage Test. The percentage of zebrafish embryos and larvae that survived was measured after exposure to different concentrations of Cr(VI), reduced Cr(VI) into Cr(III) by citric acid in water and ice, and the citric acid equivalent (Figure 5). In the control and citric acid equivalent-exposed groups, no significant abnormality was observed during embryo and larval development. Larval survival during 96 h in the control and citric acid equivalent-exposed groups was greater than 95 and 92%, respectively.

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Environ. Sci. Technol. 2022, 56, 3503−3513

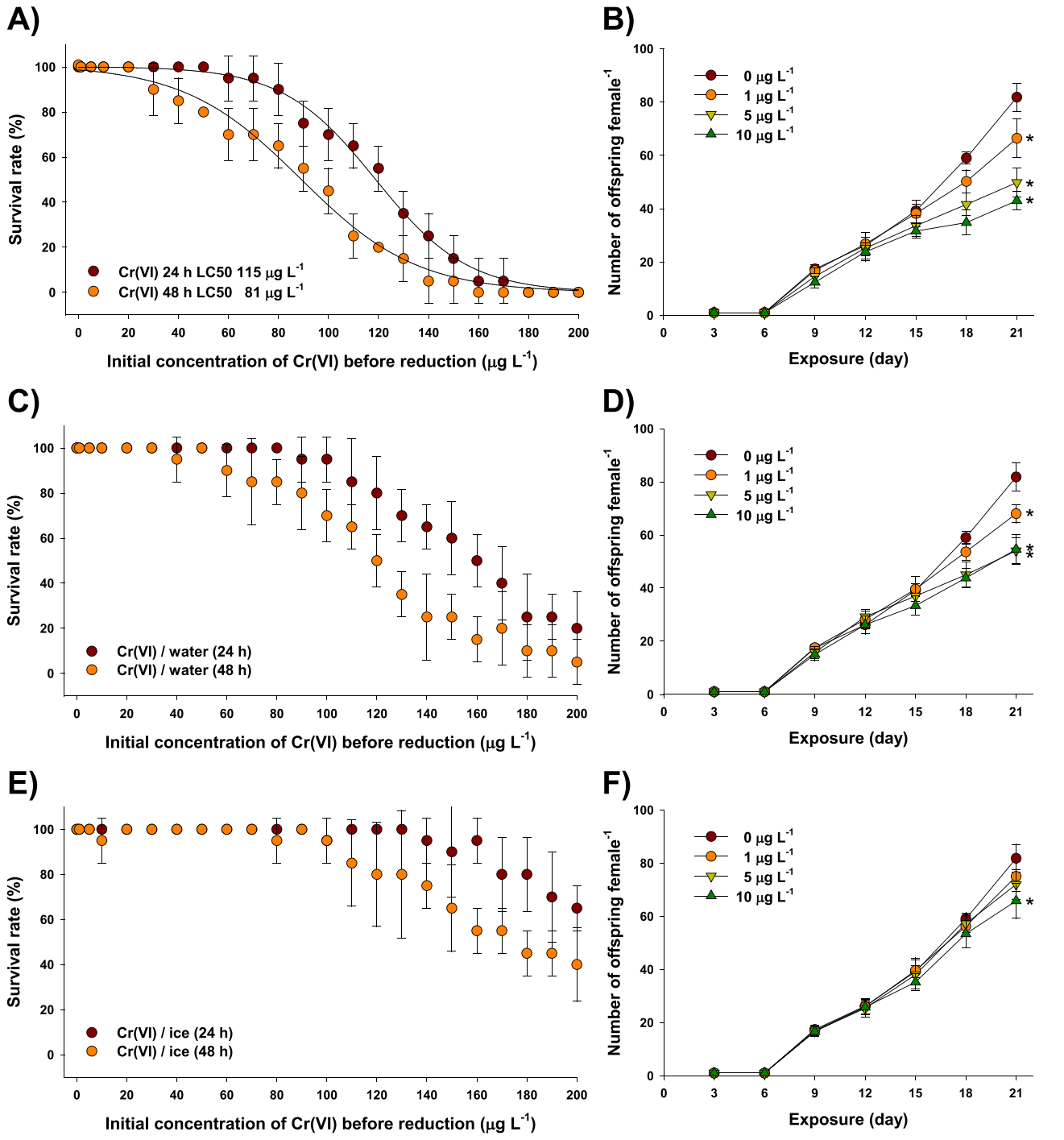

Figure 3. Results of acute toxicity and chronic reproductive effects in water fleas. (A) Measurement of 24 and 48 h survival rates of daphnids in response to Cr(VI) (0−200 μg L^{-1} at 10 μg L^{-1} intervals). (B) Cumulative number of live offspring per female for 21 days with sublethal concentrations of Cr(VI) (0−10 μg L^{-1}). (C) Measurement of the 24 and 48 h survival rate of daphnids in response to the same concentrations of Cr(VI) after reduction in water. (D) Cumulative number of live offspring per female for 21 days with sublethal concentrations of Cr(VI) after reduction in water. (E) Measurement of the 24 and 48 h survival rate of daphnids in response to the same concentrations of Cr(VI) after reduction in ice. (F) Cumulative number of live offspring per female for 21 days upon sublethal concentrations of Cr(VI) after reduction in ice. The same criteria on control and citric acid were applied in the two-generation experiment. Data are presented as the mean ± S.D. of the three groups. The asterisk (*) indicates statistical significance ($P < 0.05$) when compared with the control value.

Embryos started to hatch at 48 h (16−33%), and at 72 h, more than 97% of the embryos had hatched.

There was a significant effect of the Cr(VI) concentration on survival above 93 mg L^{-1} (Figure 5A). A drastic increase in mortality rate was observed at the transition point (72 h) from the embryo to larvae. Cumulative mortality was slightly decreased in the group exposed to Cr(VI) reduced in water compared to that in the groups exposed to Cr(VI) alone (Figure 5B). A more drastic decrease in mortality was observed in the groups exposed to Cr(VI) reduced in ice (Figure 5C). No significant mortality was observed in embryos exposed to the citric acid equivalent (Figure 5D).

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Environ. Sci. Technol. 2022, 56, 3503−3513

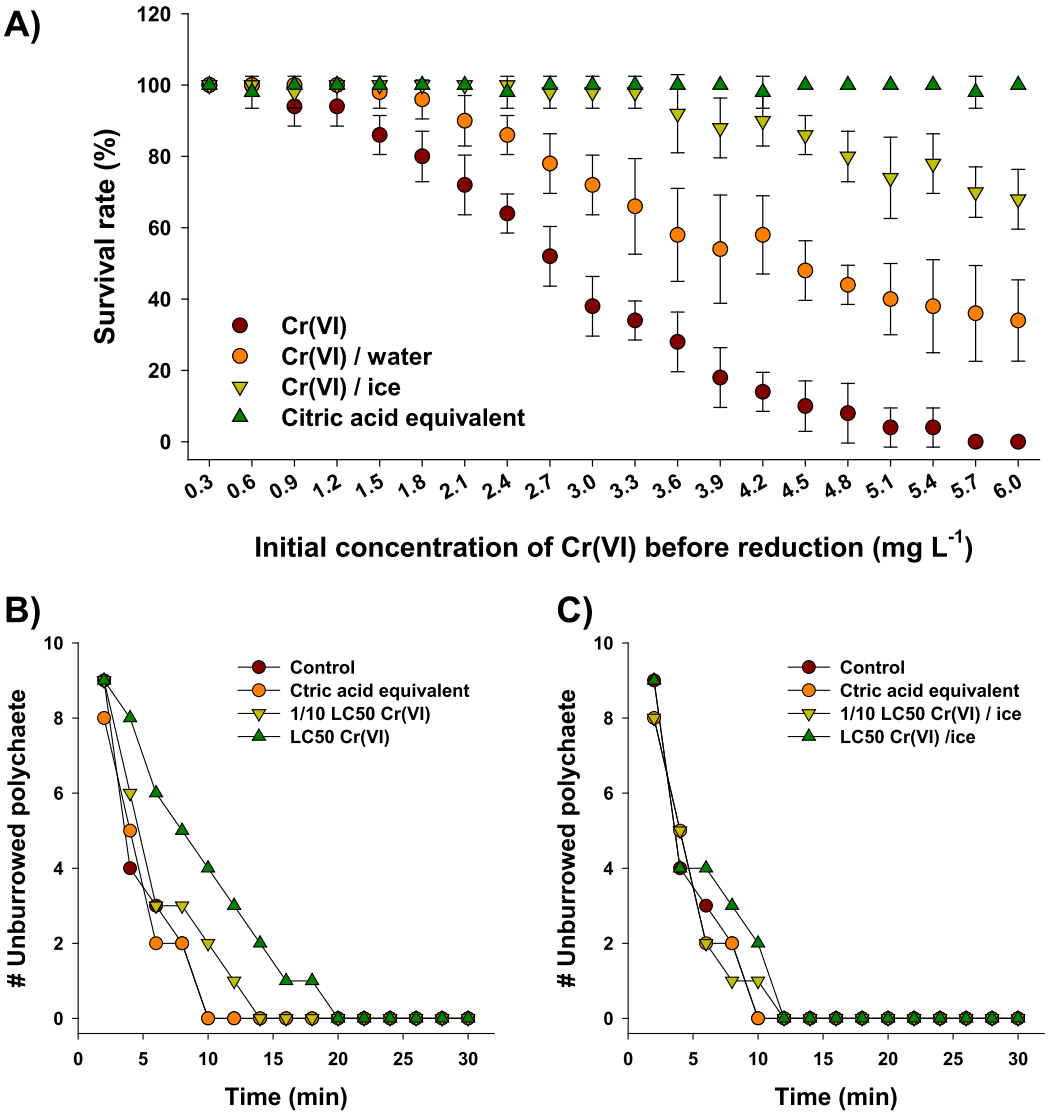

Figure 4. Results of acute toxicity in polychaetes. (A) Measurement of the 96 h survival rate of *P. aibuhitensis* in response to different concentrations of Cr(VI), Cr(VI) after reduction in water, Cr(VI) after reduction in ice, and the citric acid equivalent as a reference solvent control. Data are presented as the mean ± S.D. of the five replicates (n = 10 per replicate). (B) Effects of LC_{50} concentration of Cr(VI) and its 1/10th value on the burrowing behavior of *P. aibuhitensis*. (C) Effects of ice-based reduction of initial LC_{50} concentration of Cr(VI) and its 1/10th value on the burrowing behavior of *P. aibuhitensis*. The same criteria on control and citric acid were applied in the measurement of burrowing activity. Data are presented as the mean ± S.D. for 20 individuals.

3.7. Responses of the Antioxidant Defense System and Cholinergic Enzymes. As mortality could affect biochemical status (*e.g.*, degradation of certain protein through cell death), responses of the antioxidant defense system were measured with the toxicity values (*e.g.*, LC_{50}, mortality/survival rate) of Cr(VI) and of Cr(VI) reduced by citric acid in ice by shortening the exposure period in each animal.

A schematic overview of the response patterns and statistical significance measured in each animal is presented in Figure 6. Detailed values for each parameter are appended in Table S2 (Supporting Information). Intracellular MDA levels were significantly elevated by Cr(VI) exposure in all animals tested ($P < 0.05$), except in *Daphnia magna* ($P > 0.05$). However, no significant change in MDA levels was detected in aquatic invertebrates exposed to Cr(VI) reduced in ice ($P > 0.05$). In zebrafish, no modulation of MDA level was observed upon exposure to Cr(VI) reduced in ice during the embryo stage ($P > 0.05$), whereas a significant increase was detected during the larval stage ($P < 0.05$) (Figure 6A).

Hierarchical clustering clearly showed these differentially modulated patterns (Figure 6B). Overall, the enzymatic activity of antioxidant enzymes (*i.e.*, CAT and SOD) and the detoxification enzyme (*i.e.*, GST) showed decreased and increased patterns in response to Cr(VI) alone and Cr(VI) reduced in ice, respectively, in all the aquatic animals tested. In the case of the AChE enzyme, significantly lowered activities were observed upon Cr(VI) exposure in the amphipods, polychaete, and zebrafish ($P < 0.05$). In zebrafish, relatively long-term exposure (144 h) to Cr(VI) reduced in ice showed significantly decreased AChE activity compared to that in the control or 72 h exposed group to Cr(VI) reduced in ice ($P < 0.05$).

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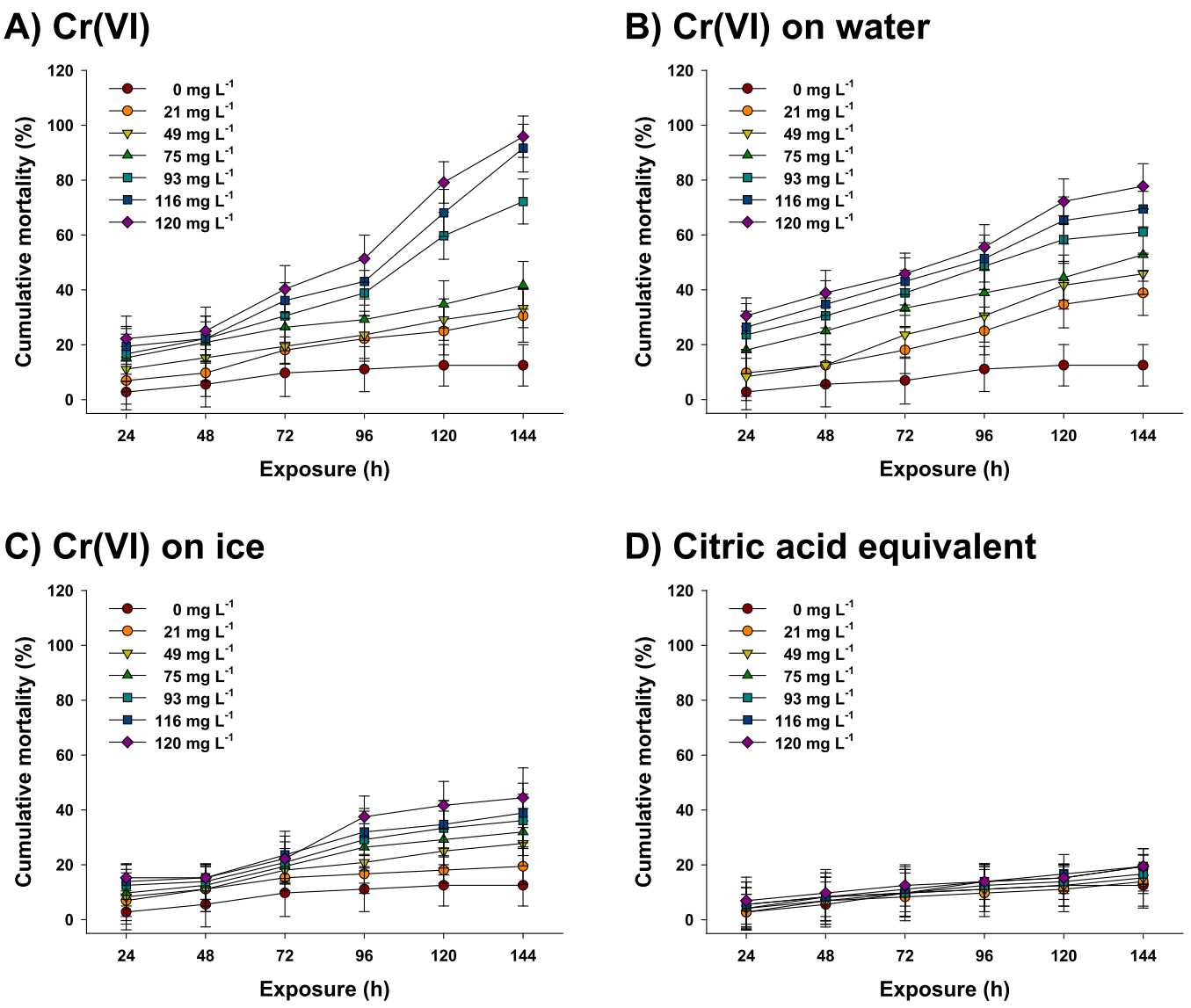

Figure 5. Results with the zebrafish early life stage. Cumulative mortality of zebrafish embryos and larvae measured in response to different concentrations of (A) Cr(VI), (B) Cr(VI) after reduction in water, (C) Cr(VI) after reduction in ice, and (D) citric acid equivalent as a reference solvent control for 144 h. The concentrations appended in B–D mean the initial concentration of Cr(VI) before the reduction process. Data are presented as the mean ± S.D. of 12 replicates (n = 6 per replicate).

4. DISCUSSION

Sensitivity to Cr(VI) and Cr(III) was high in *B. calyciflorus* than in *B. plicatilis*. The 24 h LC_{50} values observed in this study were comparable with those in previous studies conducted on rotifers. In the genus *Lecane*, the 48 h LC_{50} values ranged from 3.3 to 4.5 mg L^{-1} in response to Cr(VI).[40] Rather high tolerance was observed in Cr(VI)–exposed rotifers, such as *B. calyciflorus* (17.4 mg L^{-1}) and *Brachionus patulus* (9.2 mg L^{-1}).[41] However, significant sensitivity to Cr(VI) was also observed in the same species. The 24 h LC_{50} values were measured to be 0.004 μg L^{-1} for Cr(VI) and 640–1051 μg L^{-1} for Cr(III) in two strains of *B. calyciflorus* and 0.047 g L^{-1} for Cr(VI) and 1279 μg L^{-1} for Cr(III) in *Lecane quadridentata*.[22] These results imply that toxicity can vary with the strains in addition to species-specific sensitivity or tolerance. Experimental conditions can also affect differences between the toxicity values of Cr(VI) measured in *B. calyciflorus*; water hardness is reported to demonstrate significant modulatory potential through varied effective concentration values for Cr(VI) in the freshwater rotifer *Philodena acuticornis*.[17] Regardless of the differences in toxicity values, Cr(VI) reduction into Cr(III) in ice was found to demonstrate lower toxicity in both rotifer species.

Water fleas and amphipods have been recognized as model crustacean species for aquatic ecotoxicology and environmental research. Previously, 435 μg L^{-1} was measured as the 24 h LC_{50} value for Cr(VI) in *D. magna*.[19] Our toxicity value is more similar to a recent report, as the acute toxicity for Cr(VI) as measured in *D. magna* was 128 and 105 μg L^{-1} for 24 and 48 h, respectively.[20] The number of offspring was dose-dependently lowered by Cr(VI), suggesting the potential of a maternal effect on the second generation population of the water fleas. In the case of amphipods, the overall toxicity values of Cr(VI) and Cr(III) measured in this study were similar to the previously reported values in *H. azteca*.[21] In the previous study, 38 and 40% survival rates were measured on day 28 and 42, respectively, in response to 50 μg L^{-1} Cr(VI) exposure, whereas after exposure to 50 μg L^{-1} Cr(III), 63 and 53%

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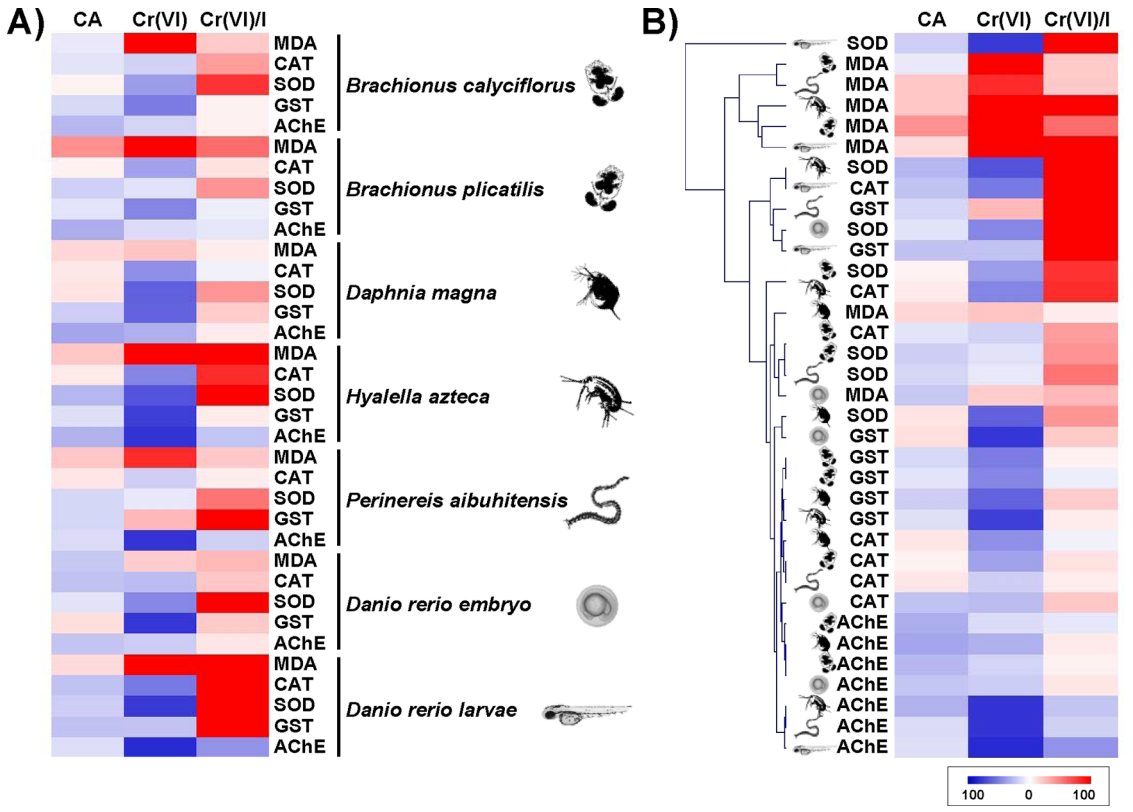

Figure 6. Schematic diagram for integrating the complete results of the biochemical parameters. (A) Results of biochemical parameters in aquatic animals exposed to Cr(VI) and reductively transformed Cr(VI) in ice for a sublethal exposure period. (B) Hierarchical clustering of the result of the biochemical parameters. The word "CA" means citric acid-treated group. The word "Cr(VI)/I" means Cr(VI) after its reduction by citric acid in the ice phase. The asterisk (*) indicates a significant difference between the control and exposed group ($P < 0.05$). Detailed information with statistical results is appended in Table S1 (Supporting Information).

survival rates were measured on day 28 and 42, respectively, with no significant differences in growth rates.[21] In both crustacean species, the Cr(VI) reduced in ice clearly showed less toxicity compared to Cr(VI) alone and that reductively transformed in water.

Polychaetes have been widely used in ecotoxicological studies on the benthic environment in estuaries and coastal areas.[42] Previously, a range of 2−7.5 mg L^{-1} was measured as the 96 h LC_{50} values of Cr(VI) in several polychaetes,[18] suggesting that polychaetes are relatively tolerant to Cr(VI) among the aquatic invertebrates tested. This study also identified its toxicity in *P. aibuhitensis* at the milligram level, which agrees well with previous findings. Another toxic effect of Cr(VI) was detected in the modulation of cholinergic activity, as delayed burrowing activity in sediment clearly suggests the neurotoxicity of Cr(VI). In *P. aibuhitensis*, mortality and retardation of burrowing activity upon Cr(VI) exposure were decreased by its reduction in ice. Taken together, these results demonstrate that the use of an ice-based platform for the reduction of Cr(VI) into Cr(III) is efficient in aquatic invertebrates.

Effective decrease of Cr(VI) toxicity by reduction into Cr(III) was also tested in an aquatic model vertebrate, zebrafish. Overall, the mortality pattern was similar to that observed in a previous study,[15] as the same experimental conditions with the same concentration of Cr(VI) were used in this study. A slight difference was observed in the hatching ratio wherein 10−29% of the eggs hatched at 48 h, and 100% of the embryos had hatched in all treatments at 72 h.[15] Drastic mortality during the transition from the embryo to the larval stage was also observed,[15] similar to our results. It is thus likely that the Cr(VI) has poor permeability in the membrane and chorion of the embryo, whereas toxicity gradually increases during larval growth from hatching. Decreased toxicity of Cr(VI) was also confirmed in zebrafish, as significant increases in survival rate were observed with ice-based reduction of Cr(VI) compared to that with Cr(VI) only and that with Cr(VI) reduced in water.

The decreased Cr(VI) toxicity by reduction into Cr(III) has been highlighted by several studies conducted in aquatic animals, as we explained. For example, the toxicity values were decreased with increasing ferrous sulfate [Fe(II)] concentrations *via* the chemical kinetics of chromate reduction by Fe(II).[20] In the present study, enhanced reduction of Cr(VI) into Cr(III) in the presence of citric acid was observed over 24 h in the ice phase. The reduction process strongly depends on the "freeze concentration phenomenon". Although the frozen sample appears fully solidified, it still contains small liquid regions between ice grain boundaries. The solutes and protons in the solution are gathered at the ice grain boundaries due to exclusion from the bulk ice crystals, and consequently, the redox conversion between Cr(VI) and citric acid occurs more rapidly within ice media than in water. To confirm the freeze concentration effect, Cr(VI) and citric acid confinement in ice grain boundaries were visualized using *in situ* confocal Raman spectroscopy. This suggested that the oxidation−reduction between Cr(VI) and citric acid occurred simultaneously within the ice grain boundaries.

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Environ. Sci. Technol. 2022, 56, 3503−3513

Although analytical evidence clearly suggests a decrease in Cr(VI) by reduction in ice, investigation of the actual modulation effects of the Cr(VI) reduced in ice at the molecular and biochemical level is still limited in aquatic animals. Thus, our measurement was expanded to obtain information regarding the potential of Cr(VI)-triggered oxidative stress using analysis of the antioxidant defense system. Based on the increased MDA level upon Cr(VI) exposure, one of the detrimental effects indicated that intracellular oxidative stress was induced by Cr(VI) treatment in the aquatic animals tested. These results support the previous findings that Cr exposure significantly modulates the oxidative status of aquatic animals.[43] Strong correlations between the antioxidant response and Cr(VI) alone or its ice-based reduction were observed in this study; Cr(VI) reduced the antioxidant capacity with induction of oxidative stress in most of the animals tested, whereas Cr(VI) reduced in ice significantly induced an antioxidant response with diminished oxidative damage. Decreased GST enzyme activity indicated that even short-term exposure to toxic values (*e.g.*, LC_{50}, mortality/survival rate) of Cr(VI) would inhibit efficient excretion and/or removal activity in aquatic animals. The results showed that exposure of aquatic animals to Cr(VI) decreased their antioxidant capacity and detoxification ability, which finally led to significant mortality. Regarding the induced patterns of the antioxidant defense system after exposure to Cr(VI) reduced in ice, aquatic animals were affected by sublethal stressful conditions and tried to maintain homeostasis by eliminating oxidative stress.

The cholinergic system plays a role in cognitive processes through acetylcholine (ACh) as the neurotransmitter and cholinergic receptors. Two cholinesterases (ChE), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), are involved in the maintenance of ACh levels with enzymatic activity.[44] As their modulations represent neurochemical alterations, ChE activity has been recognized as an important biomarker for environmental contaminants. In this study, only three species showed significant inhibition of AChE activity upon Cr(VI) treatment; overall, no significant alteration was observed in response to Cr(VI) being reduced in ice. Although the effect of metals on AChE activity is controversial, as it is unclear whether metals directly interfere with enzyme catalytic activity,[44,45] our results suggest that inhibition may result from species-specific interference or indirect pathways and that ice-based reduction can remove the potential of cholinergic modulation induced by Cr(VI).

In conclusion, our study revealed that Cr(VI) has significant detrimental effects on the physiological health status of aquatic animals at both the individual and population levels through acute mortality, impairment of growth and biochemical defense systems, and/or reduction of reproductive fitness. However, a decrease of Cr(VI) toxicity by reduction into Cr(III) in aquatic animals was successfully achieved in ice, which highlights the environmental application potential and importance of freezing-accelerated detoxification.

There are controversial reports on the higher toxicity of Cr(III) compared to Cr(VI) in certain animals,[46] with toxicity changes induced by experimental conditions such as pH and subsequent changes in the accumulation potential. As this issue was beyond the scope of our experiment, further studies are needed to highlight the effective reduction of Cr toxicity in aquatic animals. Given the importance of environmental conditions on the modulation of Cr(VI) toxicity, future studies on the effects of environmental fluctuations on toxicity variation should be prioritized to establish suitable toxicity reduction platforms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c07336.

Materials and methods; results of a toxicity test with the amphipod; results of biochemical parameters in aquatic animals; Cr(VI) concentration against [Cr(VI)]/[citric acid] ratio; time profile of Cr(VI) reduction; optical images of the concentrated Cr(VI); results on rotifer acute toxicity; and results on *Daphnia* acute toxicity and chronic reproductive effect (PDF)

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Notes

The authors declare no competing financial interest.

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