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Toxicity of Chromium (VI) to Two Mussels and an Amphipod in Water-Only Exposures With or Without a Co-stressor of Elevated Temperature, Zinc, or Nitrate

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Abstract The objectives of the present study were to develop methods for propagating western pearlshell (*Margaritifera falcata*) for laboratory toxicity testing and evaluate acute and chronic toxicity of chromium VI [Cr(VI)] to the pearlshell and a commonly tested mussel (fatmucket, *Lampsilis siliquoidea* at 20 °C or in association with a co-stressor of elevated temperature (27 °C), zinc (50 µg Zn/L), or nitrate (35 mg NO_3/L). A commonly tested invertebrate (amphipod, *Hyalella azteca*) also was tested in chronic exposures. Newly transformed pearlshell (~1 week old) were successfully cultured and tested in acute 96 h Cr exposures (control survival 100%). However, the grow-out of juveniles in culture for chronic toxicity testing was less successful and chronic 28-day Cr toxicity tests started with 4 month-old pearlshell failed due to low control survival (39–68%). Acute median effect concentration (EC50) for the pearlshell (919 µg Cr/L) and fatmucket (456 µg Cr/L) tested at 20 °C without a co-stressor decreased by a factor of > 2 at elevated temperature but did not decrease at elevated Zn or elevated NO_3. Chronic 28-day Cr tests were completed successfully with the fatmucket and amphipod (control survival 83–98%). Chronic maximum acceptable toxicant concentration (MATC) for fatmucket at 20 °C (26 µg Cr/L) decreased by a factor of 2 at elevated temperature or NO_3 but did not decrease at elevated Zn. However, chronic MATC for amphipod at 20 °C (13 µg Cr/L) did not decrease at elevated temperature, Zn, or NO_3. Acute EC50s for both mussels tested with or without a co-stressor were above the final acute value used to derive United States Environmental Protection Agency acute water quality criterion (WQC) for Cr(VI); however, chronic MATCs for fatmucket at elevated temperature or NO_3 and chronic MATCs for the amphipod at 20 °C with or without elevated Zn or NO_3 were about equal to the chronic WQC. The results indicate that (1) the elevated temperature increased the acute Cr toxicity to both mussel species, (2) fatmucket was acutely more sensitive to Cr than the pearlshell, (3) elevated temperature or NO_3 increased chronic Cr toxicity to fatmucket, and (4) acute WQC are protective of tested mussels with or without a co-stressor; however, the chronic WQC might not protect fatmucket at elevated temperature or NO_3 and might not protect the amphipod at 20 °C with or without elevated Zn or NO_3.

Native freshwater mussels, one of the most imperiled groups of animals, are in serious global decline and environmental contamination has been identified as a causal or contributing factor to these declines of mussel populations (Bogan 1993; Haag 2012; Lydeard et al. 2004; Strayer et al. 2004). In 2005, ASTM International published the standard guide for conducting laboratory toxicity tests with freshwater mussels in water exposures (ASTM 2016a). Studies conducted in accordance with the ASTM mussel testing methods have demonstrated that mussels are among the most sensitive freshwater species in the United States to a variety of contaminants, including ammonia, copper,

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nickel, zinc, chloride, sulfate, and potassium (March et al. 2007; Gillis 2011; Miao et al. 2010; Wang et al. 2007a, b, 2010, 2011, 2016a, b). However, most mussels tested are members of the family Unionidae (i.e., unionid mussels) and do not inhabit the Pacific Northwest of the United States. There has been reluctance to use toxicity data for nonnative mussel species as surrogates for mussels that inhabit the region. The Northwest possesses several endemic and phylogenetically distinctive species that are of conservation concern. These species could make acceptable test species if juvenile culture and toxicity testing methods were demonstrated to be reliable.

Western pearlshell (*Margaritifera falcata*) is a native mussel species of conservation concern in the Columbia River system, which is the largest river system in the Pacific Northwest region of North America. The Columbia River rises in the Rocky Mountains of British Columbia, Canada, flows northwest and then south into Washington State in the United States, then turns west to form most of the border between Washington and the state of Oregon before flowing into the Pacific Ocean. Historically, Hanford Reach, the longest nontidal free flowing section of the Columbia River, supported large populations of western pearlshell (Lyman 1980). However, this mussel species is now largely absent (Tiller and Marceau 2006; Mueller et al. 2011). Several mussel surveys of the Hanford Site of the Columbia River failed to detect live individuals of western pearlshell (Helmstetler 2006; Tiller and Marceau 2006), although a few shells were found that were characterized as "recently dead" based on the condition of the nacre and ligament.

The Hanford Site is located in south-central Washington State near Richland and was established in 1943 to produce nuclear materials for national defense. Several contaminants have been released from the Hanford Site to the Columbia River (Napier et al. 1995). Hexavalent chromium [Cr(VI)] was used in the reactor cooling water to retard corrosion. Chromium was discharged to the Columbia River through reactor cooling water and numerous spills that contaminated the ground, ground water, and shoreline. Recent measurements of Cr(VI) upwelling into the river from ground water were >100 µg/L in the middle of the river (Hulstrom and Tiller 2010), which was above the United States Environmental Protection Agency (USEPA) acute water quality criterion (WQC) of 16 µg/L and chronic WQC of 11 µg/L for Cr(VI) (USEPA 2016). The potential stressors at the Hanford Site include not only Cr but also other stressors, such as elevated temperature, zinc, or nitrate. There has been a concern about the thermal effects of reactor operation on aquatic organisms. A previous study has indicated that mussels in some river systems in the United States already live close to their upper thermal tolerances and, thus, might be at risk from rising environmental temperatures (Pandolfo et al. 2010). Detectable amounts of metals including copper, lead, and zinc were found in the river water and sediment samples collected near the Hanford Site (United States Department of Energy 2014). Groundwater plumes originating from irradiated material in the burial ground of the Hanford Site contained nitrate in excess of 45 mg NO_3/L and have reached the Columbia River (United States Department of Energy 2014). A field study on freshwater pearl mussel (*Margaritifera margaritifera*) in Europe found that the mortality of adult mussel significantly increased with increasing nitrate concentrations from approximately 2–13 mg NO_3/L in surface water (Bauer 1988).

Culture methods for freshwater mussels have advanced in recent decades (Barnhart 2006, Eybe et al. 2013, Scheder et al. 2014). Our work with western pearlshell is among the first efforts to culture this species, and it is necessary to evaluate whether standard toxicity testing methods are reliable with the pearlshell. In addition, the ASTM methods for conducting mussel toxicity testing need refinement. Although up to fivefold increases in dry weight of unionid mussels have been observed in chronic 28-day water tests or 28-day sediment tests, the growth of the controls has been inconsistent among tests (Ingersoll et al. 2015). Because mussel growth is often a more sensitive endpoint than survival (Besser et al. 2015; Ingersoll et al. 2015; Wang et al. 2007b, 2010, 2011, and 2013), consistency in growth across studies is important to better assess mussel responses to contaminants. Therefore, further studies were necessary to optimize feeding and other test conditions for assessing mussel growth endpoint (Ingersoll et al. 2015; Wang et al. 2011).

We conducted a series of studies to (1) develop procedures for culturing western pearlshell for conducting laboratory toxicity tests, (2) refine methods for conducting chronic water-only toxicity tests and sediment toxicity tests with juvenile pearlshell and a commonly tested unionid mussel (fatmucket, *Lampsilis siliquoidea*), and (3) determine the sensitivity of juvenile western pearlshell and fatmucket in acute and chronic water exposures to Cr(VI) with or without a co-stressor of elevated temperature, zinc, or nitrate. The present paper primarily reports the results of the acute and chronic toxicity tests. Results of this study on refining methods for conducting chronic water-only toxicity tests and sediment toxicity tests with juvenile pearlshell and fatmucket are presented in Supplemental Data, Session S1. A commonly tested benthic invertebrate (amphipod, *Hyalella azteca*) was also tested concurrently with mussels in a water-only chronic exposure. The amphipod was selected because this species was chronically sensitive to Cr(VI) (Besser et al. 2004).

Materials and Methods

Adult Western Pearlshell Collection and Juvenile Propagation

Fourteen brooding female western pearlshell were collected in early May in 2012 and 17 brooding female pearlshell were collected in late April 2013 from the South Fork Eel River, Mendocino County, CA. The mussels were shipped overnight to Missouri State University, Springfield, MO. One or two mussels were placed in one of up to nine 1 L beakers within a temperature controlled recirculating system at 11 °C in ASTM reconstituted moderate hard water (hardness 100 mg/L as $CaCO_3$ and pH 7.8; ASTM 2016b). The system was a 150 L insulated box with an attached chiller and pump. A horizontal acrylic partition divided the box into upper and lower halves and supported the 1 L beakers placed in holes through the partition with the mouths of the beakers open to the upper compartment. Water circulated through the upper compartment across the top of the beakers and to the lower compartment through a gap at the other end of the partition. Water movement within the beakers was slow so that conglutinates (aggregated eggs) remained in the beakers after release from the mussels. The mussels were monitored daily for release of conglutinates containing glochidia. Water quality was maintained by an open cell foam bio filter and daily partial water changes. The adult mussels were not fed during a 1 week holding period.

Hatchery reared rainbow trout (*Oncorhynchus mykiss*) were inoculated within 24 h of glochidia release. The glochidia isolated from 8 and more female mussels were pooled and placed in suspension at 4000 glochidia/L. Host fish were inoculated by swimming in this glochidia suspension for 30 min. The host fish were then rinsed and held in a chilled recirculating system designed for recovery of newly transformed juvenile mussels. Temperature of host fish was held at 11 °C for 7 days, 13 °C for 14 days, and 15 °C thereafter until the end of juvenile recovery. A portion of the newly transformed juveniles was shipped to the CERC for acute toxicity testing, and the remaining juveniles were cultured for 4 or 5 months (reaching 1 mm length) for use in chronic water or sediment testing.

Culture methods were adapted from those reported by Eybe et al. (2013). Newly transformed juveniles were cultured in groups of 200 in 1 L plastic sediment culture boxes with 20 mL of sieved fresh sediment (<150 μm) and 500 mL of water (5 cm deep, 20 °C). Sediment was collected biweekly from a terrestrial wetland area (Compton Hollow Conservation Area, Webster County, MO) or from the James River (Joe Crighton Access, Greene County, MO). The sediment was aerated continuously before use. No stirring or aeration was used in the culture boxes, which were left open to the air. Water was James River water (hardness 155 mg/L as $CaCO_3$, pH 8.1) with microalgae added as food (10 L of water plus 120 μL of Shellfish Diet and 200 μL of *Nannochloropsis* concentrate; Reed Mariculture, Campbell, CA). Water and sediment were changed weekly. No food was added between water changes. Culture temperature ranged from 18 to 20 °C. Unionized ammonia concentration in the water was monitored at 1- or 2-week intervals using a Seachem colorimetric meter and was below the detectable limit of 0.02 mg/L unionized ammonia nitrogen.

Culture of Fatmucket and Amphipod

Fatmucket

Gravid female fatmucket were collected from Silver Fork of Perche Creek, Boone County, MO in spring of 2012, 2013, and 2014, and held at the Columbia Environmental Research Center (CERC), US Geological Survey, Columbia, MO, in a flow-through 600-L tank with well water (hardness 300 mg/L as $CaCO_3$, alkalinity 250 mg/L as $CaCO_3$, pH 7.8) at a flow rate of 2 L/min. Water was maintained at 10–12 °C to prevent the mussels from releasing glochidia. The adult mussels were fed 20 mL of the *Nannochloropsis* concentrate and 20 mL of Shellfish Diet twice daily. The adult mussels were transferred to Missouri State University in spring or early summer for juvenile mussel culture. Roughly equal numbers of glochidia were removed from each of three adult mussels. The viability of glochidia isolated from each adult mussel was tested using the closing response to sodium chloride (ASTM 2016a). The viability of glochidia from all samples exceeded 90%. The glochidia isolated from the 3 mussels were pooled and placed on hatchery-reared largemouth bass (*Micropterus salmoides*) for metamorphosis. The host fish were maintained at 22 °C in a recirculating system equipped for recovery of juveniles. Juvenile mussels were recovered approximately 2 weeks following fish infestation. Juveniles recovered from the host fish during the 2-day peak of recovery were shipped overnight to the CERC. A portion of the juvenile mussels was used for acute toxicity testing, and the remaining juveniles were cultured for 1–2 months (reaching 1 mm length) for use in chronic toxicity testing.

Amphipod

Less than 24-h-old amphipods were isolated from the CERC in-house mass culture of mixed-age organisms using a #25 sieve (710 μm opening). A batch of 7-day-old

amphipods was cultured in-house from the <24-h-old amphipods (Ingersoll et al. 2015) for chronic toxicity testing.

Acclimation of Test Organisms

Test organisms were acclimated to test water and temperature at 20 °C (or 27 °C for the Cr test at elevated temperature) for at least 48 h (ASTM 2016a, c). The test water (70 hard water) was prepared by diluting CERC well water of hardness approximately 300 mg/L as $CaCO_3$ with deionized water to a hardness of 70 mg/L as $CaCO_3$, alkalinity of 70 mg/L as $CaCO_3$, conductivity of 200 µS/cm, pH of 8.1. The 70 mg/L hardness and 20 °C were representative of the water quality and temperature in the Hanford Reach of the Columbia River where western pearlshell exist. During the acclimation, juvenile mussels were held in 1 L glass beakers with gentle aeration through a glass pipette or in a recirculating bucket (Barnhart 2006) and amphipods were held in a 4 L container. About 25% of the water in the holding containers was gradually replaced with test water twice daily. The mussels were fed an algal mixture (*Nannochloropsis* concentrate and Shellfish Diet; Wang et al. 2007b) twice daily with an algal density of 5–10 nl cell volume/mL after each feeding. The amphipods were fed once daily for 2 day with Diatom (*Thalassiosira weissflogii*, 1200TM, Reed Mariculture, Campbell, CA) and Tetramin diet (Tetramin as suspended flakes; Ivey et al. 2016). Ambient laboratory light (~500 lx) was used with 16:8 h light:dark photoperiod during the acclimation and testing.

Preparations of Control Waters and Test Solutions

Four acute or four chronic Cr(VI) toxicity tests were conducted concurrently with a test species following standard methods outlined in ASTM International (2016a, c): (1) Cr–20 °C test (Cr toxicity alone; without a co-stressor), (2) Cr–27 °C test (Cr toxicity at elevated temperature), (3) Cr–Zn test (Cr toxicity at elevated zinc of 50 µg Zn/L), and (4) Cr–NO_3 (Cr toxicity at elevated nitrate of 35 mg NO_3/L). The temperature (20 vs. 27 °C), zinc (nominal 0 vs. 50 µg Zn/L), and nitrate (nominal 0 vs. 35 mg NO_3/L) in test waters were set at environmentally relevant levels for the Hanford Site (e.g., the upper 95th percentile for the site; the Hanford Natural Resource Trustee Council, unpublished data). Conditions for conducting the toxicity tests are summarized in Supplemental Data, Table S1.

Three waters were created as control water for the Cr tests with and without a co-stressor: (1) 70 hard water for the Cr–20 °C and Cr–27 °C tests, prepared by diluting well water a hardness of 70 mg/L as described previously, (2) 70 hard water with elevated zinc, prepared by adding zinc chloride ($ZnCl_2$, 98% purity; Sigma-Aldrich, St. Louis, MO) to the 70 hard water at 50 µg Zn/L, and (3) 70 hard water with elevated nitrate for the Cr–NO_3 test, prepared by adding sodium nitrate (Na_2NO_3, 99% purity; Sigma-Aldrich) to the 70 hard water at a concentration of 35 mg NO_3/L. Chromium (VI) oxide (CrO_3, 98% purity; Sigma-Aldrich) was used for test solution preparation. Five concentrations (with a dilution factor of 2) plus a control were tested. For acute toxicity testing under static renewal condition, the 3 control waters (70 hard water, 70 hard water with 50 µg Zn/L, or 70 hard water with 35 mg NO_3/L) were prepared and maintained in a 35 L polypropylene container at 23 °C. A solution of the highest exposure concentration was prepared in a 5 L glass jar. Fifty percent manual dilutions were performed with half of the high solution to create the lower exposure concentrations. The control water and solutions were held in the dark in a 4 °C walk-in cooler and warmed to test temperature in a water bath for use at the beginning of a test and for water renewal at 48 h. Chronic tests were conducted in intermittent flow-through proportional diluter systems modified from Mount and Brungs (1967). The three control waters were prepared and maintained in three 800 L polypropylene recirculating tanks. The Cr stock solution was delivered with each cycle of the diluter by a Hamilton syringe pump (Hamilton, Reno, NV). Each diluter delivered 5 exposure concentrations with a dilution factor of 2 plus a control.

Acute Cr Toxicity Testing

Newly transformed (5- to 8-day-old) juvenile pearlshell and fatmucket were used for acute 96 h tests conducted in June 2013 following standard methods (ASTM 2016a). At the beginning of acute tests, five pearlshell were impartially transferred into each of four 50 mL replicate glass beakers containing 30 mL of water. Test beakers were held in a plastic holding container (30 × 18 × 10 cm) with a cover to reduce evaporation. The holding containers were held in a water bath at 20 ± 1 °C (or 27 ± 1 °C for Cr–27 °C test). Approximately 75% of the water in each replicate beaker was removed and renewed on test day 2. Test organisms were not fed during acute exposures. Mussel survival (foot movement within 5 min) was determined at the end of the 96 h exposures.

Chronic Cr Toxicity Testing

A high survival of >80% was observed under control conditions in the preliminary 28-day water and sediment treatments started in October 2012 with 3-month-old pearlshell (Supplemental Data Session S1). However, in the four chronic Cr toxicity tests (Cr–20 °C, Cr–27 °C, Cr–Zn, Cr–NO_3) started in October 2013 with juvenile western

pearlshell and fatmucket, the control survival of both species was below the ASTM International test acceptability criterion of >80% control survival (ASTM 2016a). The methods and results from the 2013 chronic tests are reported in Supplemental Data Session 2.

The chronic toxicity tests were repeated in August 2014. Western pearlshell juveniles were not available for the 2014 testing because of difficulties in the culture of juvenile pearlshell for chronic testing (see "Results and Discussion" section). Instead, the repeat tests were conducted with fatmucket and amphipods following standard methods (ASTM 2016a). The four chronic toxicity tests (Cr–20 °C, Cr–27 °C, Cr–Zn, Cr–NO_3) were conducted concurrently in four diluters with juvenile fatmucket (2-month-old, 1.62 ± 0.19 mm, $n = 40$) and the amphipod (7-day-old, 1.60 ± 0.06 mm, $n = 20$). Eight 300-mL glass beakers per Cr concentration were placed in each diluter (4 beakers for fatmucket, another 4 beakers for amphipods). Each beaker had a 2.5 cm hole in the side covered with 50-mesh (279 µm width opening) stainless-steel screen and held 200 ml of water. Ten ml of silica sand (<500 µm particles; Granusil #4030, Unimin Corporation, New Canaan, CT) was added into each beaker. The diluter provided 125 ml of test solution to each replicate beaker once every 4 h (3.8 water volume additions per day). The water temperature in the diluter was maintained at 20 ± 1 °C (or at 27 ± 1 °C for Cr–27 °C test). Mussels were fed 3 mL of the algal mixture twice daily (once in early morning and once in late afternoon). The algal mixture was prepared daily, as previously described, before the morning feeding and then stored in a refrigerator at 4 °C for the afternoon feeding. The amphipods were fed once daily with 0.25 mg of diatoms and 0.5 mg of Tetramin and the feeding rate was increased over the exposure time (details in Supplemental Data Table S1).

Test beakers and sand were replaced on test day 14. Mussels and amphipods in each replicate beaker were first transferred into 200 mL glass dishes containing about 100 mL of test solution from the replicate beaker for survival determination. Mussels with empty shells or with gaped shells containing decomposed tissue, and amphipods with lack of response to gentle prodding, were classified as dead and removed from replicate beakers. Surviving mussels and amphipods were transferred into new beakers containing new sand. The sand was held in control water for 24 h before placement in exposure beakers. At the end of chronic 28-day exposures, surviving mussels in each replicate were counted and preserved in 70% ethanol to remove debris associated with the mussels before weighing. Dry weight of the mussels per replicate was determined after drying the organisms at 60 °C for 48 h. Surviving amphipods at the end of the 28-day exposures were preserved in 8% sugar formalin for subsequent length measurement and estimation of dry weight based on the empirical relationship: Weight (mg) = ((0.177* Length (mm)) − 0.0292)^3 (Kemble et al. 2013).

Water Quality and Chemical Analyses

Water temperature was monitored daily. Water quality (dissolved oxygen, pH, conductivity, hardness, alkalinity, and ammonia) were determined using standard methods (Eaton et al. 2005) on composite water samples collected from the replicate beakers in the control, medium, and high exposure concentrations at the beginning and the end of acute tests or once every week during chronic tests. Composite water samples for analyses of major cations (calcium, potassium, magnesium, and sodium) and major anions (chloride, nitrate, nitrite, and sulfate), and water samples for analyses of zinc were collected from the 3 base waters for the Cr–20 °C, Cr–Zn, and Cr–NO_3 tests at the beginning of acute exposures and biweekly during chronic exposures (the base water for the Cr–27 °C test was the same water for the Cr–20 °C test). Composite water samples for Cr analysis were collected from each of the 6 Cr concentrations at the beginning of acute tests and weekly during chronic tests. Water samples for chemical Cr and Zn analysis were collected with a polypropylene syringe fitted with a tetra-fluoroethylene sipper straw. The sample was then dispensed through a 0.45 µm pore size PES membrane filter into an acid-cleaned polyethylene bottle after discarding the first 4 mL of filtrate to rinse and equilibrate the filter cartridge. Each 20 mL sample was stabilized within 24 h by adding 0.2 mL of concentrated nitric acid. Concentrations of the metals and major cations were determined by inductively coupled plasma-mass spectrometry (Model ELAN DRC-e, PerkinElmer, Norwalk, Connecticut) in accordance with USEPA method 6020A (USEPA 2007a). The anions were quantified by ion chromatography (ICS-1100, Dionex Corporation, Sunnyvale, CA) using a method similar to USEPA method 9056A (USEPA 2007b). Water samples for analyses of dissolved organic carbon (DOC) in the control water were collected once in the middle of acute or chronic tests. Samples for DOC analysis were collected in pre-cleaned amber glass bottles, vacuum-filtered through 0.45 µm polyethersulfone membranes, and then acidified with 9 N high-purity sulfuric acid (BDH Aristar Ultra, VWR International, Radnor, PA) to pH 2 or less within 48 h of receipt. The DOC was measured as nonpurgeable organic carbon by high temperature combustion catalytic oxidation-nondispersive infrared spectroscopy using a total organic carbon analyzer (Model TOC-L CSH, Shimadzu Scientific Instruments, Inc., Columbia, MD). The method was similar to USEPA method 415.3 (USEPA 2003). Percent recoveries of Cr(VI) from reference and laboratory control solutions ranged

from 98 to 110% ($n = 7$), with an average of 102%. Percent recoveries of the cations ranged from 96 to 101%.

Established laboratory quality assurance/quality control procedures and sample types (i.e., second source calibration verification, laboratory spikes, duplicates, reference/laboratory control materials) were used to verify instrument performance, accuracy, and precision throughout the analyses. The relative percent differences between 2 duplicate analyses were <2.1% for Cr(VI), <8.6% for zinc, <0.2 for cations, <1.1% for anions. Spike recoveries ranged from 92 to 108% for Cr(VI), from 97 to 109% for zinc, from 92 to 105% for cations, from 91 to 108% for anions.

Data Analysis

Measured concentrations of Cr(VI) were used for the calculations of median effect concentrations (EC50) for survival in acute 96-h toxicity tests, and 20% effect concentrations (EC20) for survival, dry weight, biomass (total dry weight of surviving organisms per replicate) in chronic 28-day toxicity tests. The EC20s and EC50s were determined using Toxicity Relationship Analysis Program (version 1.30a, Erickson 2015). The normal probability distribution model was used for survival data analyses and logistic regression model was used for dry weight, and biomass data analyses. The exposure concentrations were log-transformed and the response of each replicate was used for the calculation. The no-observed-effect concentration (NOEC) and the lowest-observed-effect concentration (LOEC) were also determined for the endpoints in chronic toxicity tests by analysis of variance with mean comparison made by one-tailed Dunnett's test or Steel's many-one rank test using TOXSTAT® software (version 3.5, Western EcoSystems Technology). The level of statistical significance was set at $\alpha = 0.05$.

Results and Discussion

Western Pearlshell Propagation

In 2012, juvenile pearlshell were recovered between weeks 4 and 6 after inoculation of the fish hosts. About 5400 newly transformed pearlshell were recovered and cultured in the sediment culture boxes at 20 °C. Survival was approximately 70% at week 4 and 60% at week 8, and afterwards, high mortality was observed and survival decreased to 15% at week 12. At 12 weeks, shell length ranged from 0.6 to 1.2 mm (Supplemental Data, Fig. S1). In 2013, more than 23,000 juvenile pearlshell were recovered and cultured in the sediment culture boxes at 18 °C. The survival of juveniles was similar to 2012, and decreased to 10% at week 12 with a range of shell length from 0.3 to 0.7 mm. The lower growth compared to juveniles cultured similarly in 2012 may have been the result of lower culture temperature (18 °C in 2013 vs. 20 °C in 2012). The results of the 2-year mussel culture indicate that western pearlshell can be transformed in the laboratory for acute toxicity testing. However, the grow-out of juveniles in culture (reaching a suitable size of >1.0 mm for chronic water or sediment toxicity testing) was less successful. Similarly, in Europe, captive culture of *Margaritifera* species is challenging but efforts to improve methods are ongoing (Eybe et al. 2013; Scheder et al. 2014).

Water Quality and Chemical Analyses in Toxicity Tests

Water quality characteristics in acute and chronic Cr toxicity tests are summarized in Supplemental Data, Tables S2 and S3. Mean concentrations of dissolved oxygen were >6.7 mg/L) and mean concentrations of total ammonia nitrogen were <0.1 mg/L. Mean measured pH, alkalinity, hardness, and major cations and anions were similar within a test and among different acute and chronic tests (pH 7.9–8.2, alkalinity 63–75 mg/L as $CaCO_3$, hardness 71–80 mg/L as $CaCO_3$, calcium 18–23 mg/L, magnesium 6.0–7.8 mg/L, potassium 0.6–0.9 mg/L, sodium 8.3–12 mg/L, chloride 7.3–11 mg/L, and sulfate 13–18 mg/L; Tables S2 and S3). The concentration of dissolved organic carbons ranged from 0.2 to 0.4 mg C/L. The mean measured concentrations of Zn in the control water for the Cr–Zn tests ranged from 45 to 48 µg Zn/L, close to the nominal of 50 µg/L, and the mean measured concentrations of NO_3 in the control water for the Cr–NO_3 tests ranged from 35 to 39 mg NO_3/L, close to the nominal of 35 µg NO_3/L (Supplemental Data, Table S4). The control water without the addition of Zn or NO_3 contained <1.5 µg Zn/L and <0.1 mg NO_3/L. In all acute and chronic tests, measured concentrations of Cr(VI) were similar to nominal concentrations (the differences typically within 10%; Supplemental Data Table S5).

Acute Cr Toxicity to Western Pearlshell and Fatmucket

Mean control survival was 100% in all acute exposures, and met the ASTM International test acceptability criterion (TAC) of ≥90% control survival in acute exposures (ASTM 2016a). In all 4 tests with or without elevated temperature, Zn, or NO_3, acute 96-h EC50s for the pearlshell were consistently twofold or threefold greater than EC50s for fatmucket (Table 1). Within a species, the EC50 for the pearlshell (919 µg/L) or fatmucket (456 µg/L) tested at 20 °C without a co-stressor (i.e., Cr-20 °C test) was more than twofold greater than the EC50s for the pearlshell

Table 1 Acute EC50 for Cr(VI) in 96-h exposures with western pearlshell (*Margaritifera falcata*) and fatmucket (*Lampsilis siliquoidea*) at 20 °C and in association with a co-stressor of elevated temperature (27 °C), zinc (50 µg Zn/L), or nitrate (35 mg NO_3/L)

Test	EC50 (µg/L; 95% CL)	
	Western pearlshell	Fatmucket
Cr–20 °C	919 (647–1307)	456 (383–542)
Cr–27 °C	339 (275–418)	175 (150–205)
Cr–Zn	819 (678–989)	348 [248–489][a]
Cr–NO_3	997 (no CL)[b]	348 [248–489]

EC50 50% effect concentration, *CL* confidence limits

[a] An EC50 could not be calculated due to no partial mortality. A geometric mean of the bracketing concentrations with 0 and 100% mortality was calculated to obtain an estimated EC50. The 0 and 100% effect concentrations are provided in bracket as [0–100% effect concentration]

[b] *No CL* No confidence limits for EC50 estimate due to insufficient partial kills

(339 µg/L) or fatmucket (175 µg/L) tested at the elevated temperature of 27 °C, respectively, but were similar to the EC50s at elevated Zn or NO_3 (Table 1). The results indicate that (1) newly transformed juvenile pearlshell can be used for routine acute toxicity test following the ASTM mussel toxicity test methods (ASTM 2016a), (2) elevated temperature increased the acute Cr toxicity to both mussel species, but elevated Zn or NO_3 did not influence the acute Cr toxicity to either mussel, and (3) the commonly tested fatmucket was more sensitive to Cr than the pearlshell with or without a co-stressor. In another study on acute sensitivity of 5 mussel species to 10 chemicals with different modes of toxic action (Wang et al. 2016b), the EC50s for juvenile western pearlshell were about equal to (differences within a factor of 2) or greater than (a factor of >2) the EC50s for juveniles of four unionid species of different taxonomic tribes (threeridge *Amblema plicata*, paper pondshell *Utterbackia imbecillis*, fatmucket *L. siliquoidea*, and washboard *Megalonaias nervosa*).

Chronic Cr Toxicity Testing with Fatmucket and Amphipod

Mean 28-day control survival of fatmucket was 88–98% in the four chronic Cr toxicity tests with or without a co-stressor (Table 2), and met the ASTM TAC of ≥80% control survival in chronic exposures (ASTM 2016a). Mean 28-day dry weights of fatmucket in the controls (0.32–0.37 mg) were similar among the three tests at 20 °C with or without elevated Zn or NO_3 whereas the mean dry weight of fatmucket in the control (1.35 mg) tested at 27 °C was fourfold greater (Table 2). However, the maximum acceptable toxicant concentrations (MATC; geometric mean of the NOEC and LOEC) for mussels at 20 °C (26 µg/L) decreased by a factor of approximately 2 at elevated temperature or elevated NO_3 but did not decrease at elevated Zn (Table 2). No significant reduction in dry weight or biomass was observed at any concentrations which did not cause significant reduction in survival (Table 2).

Mean 28-day control survival of amphipods was 83–98% in the four chronic Cr toxicity tests (Table 2) and met the TAC of ≥80% control survival in chronic exposures (ASTM 2016c). In the Cr–Zn test, however, the amphipods in all four control replicates grew poorly and the mean dry weight was only 0.06 mg, which was eightfold less than the mean dry weights of control amphipods (0.48–0.54 mg) in other three Cr tests (Cr–20 °C, Cr–27 °C, or Cr–NO_3), and was even three to sevenfold less than the dry weights in the five treatments of the Cr–Zn test (Table 2). It is not clear why the growth of amphipods was slow in the control water with 50 µg Zn/L. Limited chronic Zn toxicity data are available for the amphipod (*H. azteca*). In a 10-week exposure with dechlorinated city tap water with 130 mg/L hardness (Borgmann et al. 1993), no effect on amphipod growth (wet weight) was observed at the concentrations up to 316 µg Zn/L. In addition, the amphipod growth (dry weight) was not affected at the concentrations up to 100 µg Zn/L in a 42-day exposure in diluted well water to a hardness of 100 mg/L (James Kunz, unpublished data). The results from the other studies indicate the concentration of 50 µg Zn/L unlikely resulted to the poor growth of amphipods although the present study was conducted at a lower hardness (70 mg/L). Nevertheless, the potential effect of the hardness on Zn toxicity needs to be evaluated. In contrast to amphipods in the control, the amphipods in the low Cr treatment (2.8 µg Cr/L) grew well (mean dry weight 0.45 mg) in the Cr–Zn test (~8-fold greater than the control; Table 2). The possibility of Cr–Zn interaction may also need to be evaluated. Ince et al. (1999) assessed the interactive toxicity of several metals in binary mixture using a battery of Microtox test with *Vibrio fisheri* and duckweed test with *Lemna minor*, and found that the Zn–Cr(VI) interaction was additive based on Microtox test results but antagonistic based on duckweed test results. In addition, the mussel test results from the present study indicate that the Cr toxicity showed no Zn–Cr interaction (Table 2). Because of the uncertainty in the control growth data, the effect concentrations based on the dry weight and biomass from the Cr–Zn test were not estimated (Table 2).

The MATCs for amphipod dry weight or biomass were lower than MATCs for amphipod survival in the Cr–20 °C and Cr–NO_3 tests (Table 2). Based on the most sensitive endpoint, the MATC for amphipods at 20 °C did not decrease at elevated temperature, Zn or NO_3 (Table 2). Compared with the effect concentrations for fatmucket, the

Table 2 Mean responses ($n = 4$, standard deviation in parenthesis) of fatmucket (*Lampsilis siliquoidea*) and amphipod (*Hyalella azteca*) in the 2014 chronic 28-day Cr(VI) toxicity tests in association with a co-stressor of elevated temperature (27 °C), zinc (50 μg Zn/L), or nitrate (35 mg NO_3/L) and chronic effect concentrations based on lethal and sublethal endpoints in each test

Nominal conc. (μg Cr/L)	Cr–20 °C				Cr–27 °C				Cr–Zn				Cr–NO_3			
	Measured conc. (μg Cr/L)	Survival (%)	Dry weight[a] (mg)	Biomass (mg)	Measured conc. (μg Cr/L)	Survival (%)	Dry weight[a] (mg)	Biomass (mg)	Measured conc. (μg Cr/L)	Survival (%)	Dry weight[a] (mg)	Biomass (mg)	Measured conc. (μg Cr/L)	Survival (%)	Dry weight[a] (mg)	Biomass (mg)
Fatmucket																
Control	0.3 (0.3)	93 (5.0)	0.35 (0.15)	3.18 (1.40)	0.5 (0.3)	88 (5.0)	1.35 (0.16)	11.9 (1.9)	0.5 (0.4)	98 (5.0)	0.37 (0.06)	3.58 (0.56)	0.5 (0.3)	93 (5.0)	0.32 (0.09)	2.95 (0.74)
2.5	–[b]	–	–	–	2.7 (0.4)	90 (12)	1.36 (0.34)	12.0 (1.6)	2.8 (0.3)	93 (9.6)	0.38 (0.02)	3.49 (0.42)	2.7 (0.2)	85 (13)	0.25 (0.04)	2.11 (0.29)
5.0	5.0 (0.5)	93 (15)	0.37 (0.03)	3.46 (0.74)	5.0 (0.4)	90 (0.0)	1.64 (0.27)	14.7 (2.4)	5.0 (0.4)	100 (0.0)	0.35 (0.09)	3.52 (0.96)	5.0 (0.3)	85 (13)	0.24 (0.07)	2.04 (0.63)*
10	9.2 (0.5)	85 (10)	0.32 (0.08)	2.76 (0.85)	10 (0.5)	80 (14)	1.76 (0.54)	13.8 (4.4)	9.7 (0.5)	100 (0.0)	0.33 (0.08)	3.24 (0.79)	10 (0.5)	90 (8.2)	0.27 (0.06)	2.45 (0.58)
20	19 (1.0)	95 (5.8)	0.38 (0.05)	3.58 (0.38)	21 (1.0)	28 (25)*	*1.80 (1.20)*[c]	4.4 (3.9)*	19 (0.6)	98 (5.0)	0.34 (0.11)	3.31 (1.21)	20 (0.4)	68 (15)*	*0.16 (0.05)*	1.11 (0.57)*
40	36 (0.6)	10 (12)*	*0.21 (0.05)*[c]	0.20 (0.24)*	42 (1.0)	0.0 (0.0)*	NA	0.0 (0.0)*	42 (1.0)	85 (13)*	*0.25 (0.03)*	2.10 (0.53)*	42 (1.0)	7.5 (9.6)*	*0.12 (0.08)*[c]	0.07 (0.09)*
80	84 (1.0)	2.5 (5.0)*	*0.12* (NA)[c]	0.03 (0.06)*	–	–	–	–	–	–	–	–	–	–	–	–
NOEC		19	19	19		10	10	10		19	19	19		10	10	10
LOEC		36	>19	36		21	>10	21		42	>19	42		20	>10	20
MATC		26	NC	26		14	NC	14		28	NC	28		14	NC	14
Amphipod																
Control	0.3 (0.3)	95 (5.8)	0.54 (0.11)	5.18 (1.34)	0.5 (0.3)	98 (5.0)	0.48 (0.04)	4.64 (0.47)	0.5 (0.4)	83 (15)	0.06 (0.02)	0.50 (0.06)	0.5 (0.3)	98 (5.0)	0.50 (0.07)	4.88 (0.62)
2.5	–	–	–	–	2.7 (0.4)	80 (18)	0.42 (0.05)	3.31 (0.72)	2.8 (0.3)	80 (8.2)	0.45 (0.08)	3.57 (0.58)	2.7 (0.2)	83 (24)	0.47 (0.06)	3.93 (1.42)
5.0	5.0 (0.5)	100 (0.0)	0.54 (0.03)	5.36 (0.28)	5.0 (0.4)	93 (9.6)	0.44 (0.03)	4.11 (0.52)	5.0 (0.4)	72 (15)	0.36 (0.05)	2.56 (0.61)	5.0 (0.3)	98 (5.0)	0.09 (0.04)*	0.81 (0.39)*
10	9.2 (0.5)	93 (5.0)	0.54 (0.10)	5.02 (0.99)	10 (0.5)	93 (9.6)	0.45 (0.09)	4.18 (0.86)	9.7 (0.5)	65 (10)	0.32 (0.08)	2.14 (0.75)	10 (0.5)	95 (5.8)	0.41 (0.07)	3.93 (0.83)
20	19 (1.0)	88 (13)	0.44 (0.11)	3.74 (0.71)*	21 (1.0)	85 (13)	0.45 (0.14)	3.89 (1.53)	19 (0.6)	55 (13)*	0.13 (0.11)	0.79 (0.81)	20 (0.4)	98 (5.0)	0.23 (0.16)*	2.21 (1.61)*
40	36 (0.6)	80 (23)	0.06 (0.02)*	0.50 (0.26)*	42 (1.0)	65 (13)*	*0.53 (0.04)*	3.45 (0.67)	42 (1.0)	33 (15)*	0.22 (0.04)	0.70 (0.36)	42 (1.0)	65 (19)*	*0.44 (0.12)*	2.77 (0.78)*
80	84 (1.0)	5.0 (5.8)*	*0.07 (0.09)*[c]	0.07 (0.09)*	–	–	–	–	–	–	–	–	–	–	–	–
NOEC		36	19	9.2		21	21	42		10	42	42		20	10	10
LOEC		84	36	19		42	>21	>42		19	>42	>42		42	20	20
MATC		55	26	13		30	NC	NC		14	NE[d]	NE[d]		29	14	14

NOEC no-observed-effect concentration, *LOEC* lowest-observed-effect concentrations, *MATC* maximum acceptable toxicant concentration (expressed mathematically as geometric mean of the NOEC and LOEC). *EC20* 20% effect concentration, *CL* confidence limits, *NC* not calculated, *NE* not estimated, *NA* not applicable

An asterisk (*) indicates significant reduction relative to the control. Dry weight data at the concentrations above the NOEC for survival (mean in italics) were excluded from hypothesis testing for calculating the LOEC

[a] Initial dry weight at the beginning of exposures: fatmucket 0.20 ± 0.04 mg ($n = 4$ replicate means); amphipod 0.018 ± 0.002 mg ($n = 20$ individuals)

[b] This exposure concentration was not tested in this test

[c] $n = 1$–3 due to 100% mortality in 1–3 replicates at the high exposure concentration

[d] Effect concentration was not estimated because the amphipod growth in the control was unusually low (see text for details)

MATC for amphipod was twofold less at 20 °C without a co-stressor and at the elevated Zn but were equal to or greater at elevated NO_3 or temperature (Table 2).

Chronic EC20 were estimated for mussels and amphipods in the chronic tests. The EC20s generally had broad or no confidence limits and in some cases could not be estimated, because the toxicity data did not meet the conditions for any regression analysis. Therefore, the EC20s are presented in Supplemental Data Table S6, as supporting information. Noticeable, the EC20s were generally similar to the MATCs described previously (Supplemental Data Table S6).

The results of the chronic tests with fatmucket and amphipods indicate that, for fatmucket, the elevated temperature or elevated NO_3 increased chronic Cr toxicity but the elevated Zn did not influence the Cr toxicity. However, for amphipods, the elevated Zn and NO_3 did not influence the Cr toxicity at 20 °C; in fact, the elevated temperature of 27 °C reduced the Cr toxicity to amphipods at 20 °C. The results also indicate that the amphipod is equally or more sensitive than fatmucket in the chronic exposures with or without a co-stressor of Zn or NO_3. The high sensitivity of the amphipod was consistent with the finding in a previous study. Besser et al. (2004) reported a MATC of 14 µg/L in 28- and 42-day Cr(VI) exposures conducted with amphipods (*H. azteca*) at 23 °C. The MATC from Besser et al. (2004) was calculated based on survival (no dry weight and biomass were determined) and was lower than the MATC for survival (55 µg/L) but close to the MATC for biomass (13 µg/L) at 20 °C obtained from the present study (Table 2).

It is interesting that the amphipod sensitivity to Cr decreased with increasing temperature from 20 to 27 °C and the control growth was substantially lower in test water with elevated Zn (Table 2). Further studies are needed to evaluate the influence of temperature and Zn concentration in test water on chronic Cr toxicity to amphipods, such as, testing the amphipod a standard test temperature of 23 °C for amphipods (ASTM 2016c) and at a lower concentration of Zn (e.g., 30 µg/L, rather than 50 µg/L used in the present study).

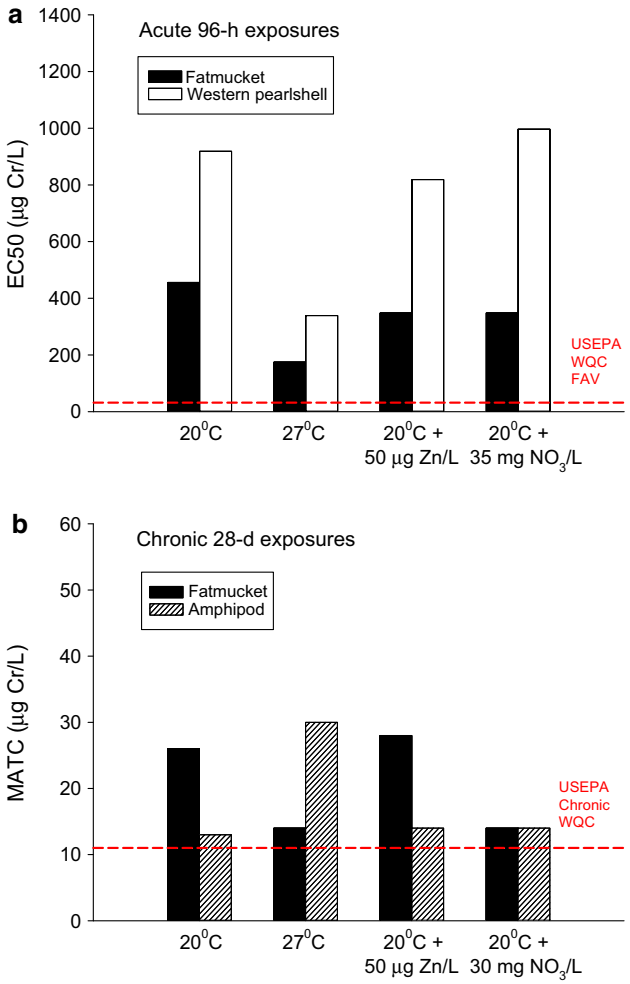

Fig. 1 Acute 50% effect concentration (EC50) and chronic maximum acceptable toxicant concentration (MATC) in acute and chronic Cr(VI) exposures with western pearlshell (*Margritifera falcata*), fatmucket (*Lampsilis siliquoidea*), and amphipod (*Hyalella azteca*) at 20 °C or in association with a co-stressor of elevated temperature (27 °C), zinc (50 µg Zn/L), or nitrate (35 mg NO_3/L). *Dash line* indicates the Unites States Environmental Protection Agency (USEPA) final acute value (FAV) or chronic water quality criterion (WQC) for Cr(VI)

Protection of Ambient Water Quality Criteria for Cr(VI)

Acute EC50s for both mussel species in the 4 acute exposures with or without a co-stressor were far above the final acute value of 32 µg/L used to derive the USEPA acute WQC for Cr(VI) (acute criterion = ½ final acute value; Fig. 1a). However, at elevated temperature or NO_3, the 28-day MATCs for fatmucket from the 2014 chronic Cr test (Fig. 1b) were about equal to the chronic WQC of 11 µg/L. The 28-day MATCs for amphipod at 20 °C (without a co-stressor) and at elevated Zn or NO_3 were also about equal to the chronic WQC (Fig. 1b).

The chronic WQC for Cr(VI) was estimated from an acute-chronic ratio Because insufficient chronic toxicity data were available to calculate final chronic value using 8-family procedure, the chronic WQC for Cr(VI) was estimated from an acute-chronic ratio (USEPA 1995). Chronic effect concentrations in the WQC Cr database ranged from 6.1 to 40 µg/L for aquatic invertebrates and from 265 to 2,213 µg/L for fish (USEPA 1995). Including the amphipod and fatmucket from the presented study would rank both species among the four most sensitive species in the chronic Cr toxicity database.

Conclusions

Newly transformed juvenile western pearlshell were successfully cultured in the laboratory over two seasons for acute toxicity testing, but the grow-out of juveniles in culture (reaching a suitable size of >1.0 mm for chronic water and sediment toxicity testing) was less successful. Acute 96-h Cr toxicity tests were successfully completed with newly transformed pearlshell following standard methods (ASTM 2016a). High survival of >80% was observed under control conditions in preliminary 28-day water and sediment treatments started with 3-month-old pearlshell (Supplemental Data Session S1), indicating that the pearlshell can be successfully tested in a 28-day water-only or sediment exposures. However, in subsequent chronic 28-day water-only Cr toxicity test, the control survival of the pearlshell was below the ASTM International test acceptability criterion of >80% control survival, likely due to poor quality of test organisms. The results of acute toxicity tests indicate that the commonly tested fatmucket was acutely more sensitive to Cr than the pearlshell in water with or without a co-stressor of elevated temperature, Zn, or NO_3, and thus, can be a surrogate to be protective of acute Cr toxicity to the pearlshell. Elevated temperature increased the acute Cr toxicity to both mussel species but elevated Zn or NO_3 did not. Four chronic Cr toxicity tests with or without elevated temperature, Zn, or NO_3 were successfully completed with the fatmucket and amphipod. The elevated temperature or elevated NO_3 increased chronic Cr toxicity to fatmucket, whereas the elevated temperature, Zn, or NO_3 did not increase Cr toxicity to the amphipod. The amphipod was equally or more sensitive than fatmucket in chronic exposures at 20 °C with or without elevated Zn or NO_3. The USEPA WQC for Cr(VI) protect western pearlshell and fatmucket in acute Cr exposures with or without elevated temperature, Zn, or NO_3, but may not adequately protect fatmucket (perhaps other mussels including western pearlshell) with elevated temperature or NO_3 in chronic Cr exposure. In addition, the WQC may not adequately protect the amphipod at 20 °C with or without elevated Zn or NO_3 in chronic exposures.

In recent study at our laboratory to refine mussel culture methods, western pearlshell showed improved survival and growth in a flow-through, auto-feeding system (James Kunz, unpublished data). While it is now possible to successfully culture and retest western pearlshell in chronic Cr exposures using the refined method, our work with the pearlshell indicates that glochidia collection for juvenile culture are available only during approximately 2 months of the year because the pearlshell is a short-term brooder which provides the short-term production of only one brood of young per year. Therefore, it is necessary to consider surrogate mussel species for toxicity testing. Other native mussels in the Columbia River, such as Oregon floater (*Anodonta oregonensis*) and California floater (*A. californiensis*), have been successfully cultured in our laboratories (Chris Barnhart, unpublished data). The *Anodonta* species are long-term brooders, which allow extended animal availability throughout the year to be used in culture and toxicity testing. In addition, our culture studies indicate that the *Anodonta* species grow much faster than the pearlshell, and thus, facilitate measurement of effects on growth.

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