RELATIVE SENSITIVITY OF AN AMPHIPOD HYALELLA AZTECA, A MIDGE CHIRONOMUS DILUTUS, AND A UNIONID MUSSEL LAMPSILIS SILIQUOIDEA TO A TOXIC SEDIMENT

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(Submitted 6 November 2014; Returned for Revision 15 September 2015; Accepted 25 January 2015)

Abstract: The objective of the present study was to evaluate the relative sensitivity of test organisms in exposures to dilutions of a highly toxic sediment contaminated with metals and organic compounds. One dilution series was prepared using control sand (low total organic carbon [TOC; <0.1%, low binding capacity for contaminants]) and a second dilution series was prepared using control sediment from West Bearskin Lake, Minnesota, USA (high TOC [~10% TOC, higher binding capacity for contaminants]). Test organisms included an amphipod (Hyalella azteca; 10-d and 28-d exposures), a midge (Chironomus dilutus; 20-d and 48-d exposures started with <1-h-old larvae, and 13-d and 48-d exposures started with 7-d-old larvae), and a unionid mussel (Lampsilis siliquoidea; 28-d exposures). Relative species sensitivity depended on the toxicity endpoint and the diluent. All 3 species were more sensitive in sand dilutions than in West Bearskin Lake sediment dilutions. The <1-h-old C. dilutus were more sensitive than 7-d-old C. dilutus, but replicate variability was high in exposures started with the younger midge larvae. Larval biomass and adult emergence endpoints of C. dilutus exhibited a similar sensitivity. Survival, weight, and biomass of H. azteca were more sensitive endpoints in 28-d exposures than in 10-d exposures. Weight and biomass of L. siliquoidea were sensitive endpoints in both sand and West Bearskin Lake sediment dilutions. Metals, ammonia, oil, and other organic contaminants may have contributed to the observed toxicity. Environ Toxicol Chem 2015;34:1134–1144. © 2015 The Authors. Published by SETAC.

Keywords: Sediment toxicity Benthic macroinvertebrates Sediment quality guideline Oil Metals

INTRODUCTION

Methods for conducting whole-sediment toxicity tests with freshwater sediments were initially developed in the 1980s and 1990s for the amphipod Hyalella azteca, for the midge Chironomus dilutus [1–4], and for oligochaetes, mayflies, cladocerans, and other species of amphipods and midges [1,5,6]. Endpoints measured in H. azteca sediment toxicity tests include 10-d to 28-d survival, weight, biomass, and 42-d adult reproduction [1,2]. Endpoints measured in C. dilutus sediment toxicity tests include 10-d to 20-d larval survival, weight, biomass, and 60-d emergence and reproduction of adult flies. The ASTM International [1] and US Environmental Protection Agency (USEPA) [2] sediment toxicity methods rated freshwater mussels as low priority for whole-sediment toxicity testing primarily because of the lack of methods for culturing or conducting toxicity tests with mussels and because of limited information on the sensitivity of mussels to contaminants of concern in sediment (see rankings provided in Table 1.3 in the USEPA methods [2]).

In the past decade, methods for conducting water-only toxicity tests were developed for a variety of species and life stages of freshwater mussels [7]. Toxicity endpoints for mussels include 24-h larval (glochidia) survival, 96-h juvenile survival, and 28-d juvenile survival, length, weight, and biomass. Water-only toxicity testing has demonstrated that juvenile mussels are sensitive to a variety of contaminants associated with sediment including metals and ammonia, but may be less sensitive to organic contaminants compared with commonly tested species [8–13]. Moreover, juvenile mussels are in direct contact with sediment, often burrowing into the upper 1-cm to 2-cm layer of sediment [14]. Water-only toxicity methods for mussels [7] have been adapted to conduct 28-d whole-sediment toxicity tests with freshwater mussels [15–17]. However, additional data are needed comparing the response of mussels with other commonly tested species in whole-sediment toxicity tests.

The objective of the present study was to evaluate relative sensitivity of commonly tested H. azteca and C. dilutus to a unionid mussel (Lampsilis siliquoidea) with exposure to dilutions of a highly contaminated and highly toxic field-collected sample (comparing different life stages, durations of exposure, or toxicity endpoints). Lampsilis siliquoidea was selected for testing because this species of mussel has been cultured and tested routinely in water-only toxicity tests, has been demonstrated to be sensitive to contaminants typically of concern in sediment, and exhibits a sensitivity similar to that of other freshwater mussels [9–13]. The sediment was collected from the East Branch of the Grand Calumet River (located south of Lake Michigan, USA, and contained elevated concentrations of metals and organic compounds). The Grand Calumet River sediment was diluted with a high total organic carbon (TOC) control sediment collected from West Bearskin Lake in Minnesota (~10% TOC, similar to the site sediment [18] with a higher binding capacity for the contaminants) and was diluted with a low TOC sand (<0.1% TOC, with a lower binding capacity for the contaminants).
Sediment collection, preparation, and characterization

Composite sediment samples were initially collected from 6 Grand Calumet River sites in March 2011 by the USEPA Great Lakes National Program Office (Chicago, IL, USA). Approximately 50 L of sediment was collected from the sediment surface at each site and was held in the dark at 4 °C before testing. The sampling sites were located between Kennedy Avenue and State Highway 912, north of Interstate I-90 (approximately 41°36′56.54″N by 87°26′49.20″W; Supp lmental Data, Figure S1). Previous 10-d toxicity testing was conducted with the undiluted Grand Calumet River sediment within 1 mo of sediment collection with H. azteca and with C. dilutus (Anonymous, Great Lakes Environmental Center, Traverse City, MI, USA; unpublished data). All 6 samples reduced survival, weight, or biomass of test organisms relative to a control sediment and had elevated concentrations of metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and oil range organics estimated as diesel range organics and residual range organics (Anonymous, Great Lakes Environmental Center, Traverse City, MI, USA, unpublished data). One of the 6 Grand Calumet River sediments, the SS-05 sediment sample (Table 1; Supplemental Data, Figure S1), was selected to create dilutions for toxicity testing in the present study based on the relatively high concentration of contaminants of concern in the whole sediment samples, moderate concentrations of ammonia in porewater, and 100% mortality to H. azteca and to C. dilutus in the previous 10-d exposures.

The chemical characteristics of the 100% Grand Calumet River sediment were: total PCBs, 2.8 μg/g (as aroclor 1248); total PAHs, 71 μg/g (16 analytes or 182 μg/g for 35 analytes); oil range organics, 35 mg/g; simultaneously extracted metals minus acid volatile sulfide (SEM-AVS), 197 μmole/g; SEM-AVS normalized to gram organic carbon, 197 μmole/g; AVS, 32 μmole/g (/19; Table 1). The chronic sum equilibrium-partitioning sediment benchmark toxic unit (ΣESBTU) for 100% Grand Calumet River sediment sample was 2.46 μmole total PAHs/g organic carbon in sediment (estimated for 34 parent and alkylated PAHs; [20], see Data Analysis Section below).

Probable effect concentration quotients (PECQs) calculated by dividing chemical concentrations in whole sediment by the probable effect concentration [21] for the 100% Grand Calumet River sediment sample were: 4.14 for total PCBs, 3.11 for total PAHs (16 analytes), and 9.77 for mean metals PECQs (based on As, Cd, Cr, Cu, Pb, Ni, Zn), for an overall mean PECQ of 5.67 (based on PECQs for total PCBs, total PAHs, and mean metals) [21]. The physical characteristics of the 100% Grand Calumet River sediment were: dry weight, 48%; density, 1.66 g/mL; TOC, 9.8%; gravel, 10%; sand, 33%; silt, 49%; and clay, 8%. Historic chemical characteristics of West Bears kinase sediment are, approximately: total PCBs, 0.053 μg/g (as aroclor 1248); PAHs, 0.1 μg/g (15 analytes); ΣESBTUs, 0.02 μmole total PAHs/gc (estimated from 15 PAHs); oil range organics, <0.1 mg/g; AVS, approximately 40 μmole/g; SEM-AVS, approximately 39 μmole/g; SEM-AVS/gc, approximately 390 μmole/g and PECQ for total PCBs of 0.08, for total PAHs of 0.02, for mean metals of 0.24, and overall mean PECQ of 0.11 [18,22].

Historical physical characteristics of West Bears kinase sediment are approximately: dry weight, 17%; density, 1.66 g/mL; TOC, 10.3%; sand, 74%; silt 10%; and clay, 16%. The quartz sand was obtained as Granulsi #4030 from Unimin Corporation and was sieved to <0.5 mm (likely <0.1% TOC). This source of sand has been routinely used as a control
substrate by both the US Geological Survey Columbia Laboratory (CERC) and the USEPA Duluth Laboratory and has been used in inter-laboratory testing conducted with *H. azteca* (D.R. Mount and C.G. Ingersoll, unpublished data).

Dilutions of Grand Calumet River sediment (0%, 6.25%, 12.5%, 25% and 50%) were prepared on a dry weight basis of sediment particles using either sand as the diluent (low TOC) or West Bearskin Lake sediment as the diluent (high TOC similar to the TOC in the 100% Grand Calumet River sediment). Concentrations of contaminants were estimated in the dilutions of Grand Calumet River sediment based on dry weight concentrations in the 100% Grand Calumet River sediment sample or based on dry weight concentrations in the West Bearskin Lake sediment sample assuming no contribution of contaminants from sand (Table 1). The concentrations of total PCBs, total PAHs and metals, and mean PECQs on a dry weight basis would be expected to decrease proportionally with increasing proportion of sand or West Bearskin Lake sediment. The concentration of total PAHs based on ΣESBTU would be expected to decrease with increasing addition of West Bearskin Lake sediment, given that ΣESBTUs are normalized to TOC and the TOC was similar between the Grand Calumet River sediment and West Bearskin Lake sediment (Table 1). The estimated ΣESBTUs would expected to remain relatively constant in dilutions with sand, however, given that the concentrations of PAHs and TOC would be proportionally diluted with sand that had virtually no TOC.

The dilutions of sediment were prepared in August 2011 by weighing and mixing wet subsamples of the 100% Grand Calumet River sediment with wet subsamples of West Bearskin Lake sediment or with wetted sand (Supplemental Data, Table S1). The components for a dilution treatment were prepared by first adding the appropriate wet mass (estimated from dry mass) of each of the homogenized sediments or sand into 2.5-L plastic buckets, and then mixing for 3 min with a stainless-steel auger and hand-held drill (scraping the bottom of the bucket with a spoon about every minute during the mixing period). The auger diameter was 7.6 cm with an overall length of 38 cm and an auger bit length of 25.4 cm (Augers Unlimited [18]). Diluted sediments and control sediments were stored in the dark at 4°C for 3 wk and were re-homogenized weekly with the stainless steel auger.

Before the start of the sediment toxicity tests, each sediment sample was re-homogenized with the auger and placed into exposure beakers, and overlying water was added (~7 d [test day –7] before test organisms were placed in the sediment exposure beakers). The sediments in the exposure beakers were held for approximately 7 d under static conditions to allow the sediment samples to better equilibrate before the start of the exposures [15–17].

On test day –7, subsamples of sediments were prepared for measuring porewater quality (ammonia, pH, free sulfide, hardness, alkalinity, conductivity, and dissolved oxygen [23]). Subsamples of sediment for isolating porewater were centrifuged at approximately 5200 rpm (7000 g) for 15 min at 4°C. Approximately 20 mL to 50 mL of porewater was used to measure ammonia, and a similar volume of porewater was used to measure the other water quality characteristics. The concentration of total ammonia nitrogen (N) was determined with an Orion ammonia electrode and Orion EA940 meter (Thermo Electron). The concentrations of un-ionized ammonia nitrogen were calculated based on measured total ammonia, pH, and temperature [24]. Hydrogen sulfide was estimated from total dissolved sulfide at the pH of each porewater sample using the conditional ionization constant for hydrogen sulfide in fresh water [23].

In both the sand dilution series and the West Bearskin Lake sediment dilution series, porewater hardness, alkalinity, conductivity, sulfate, sodium, potassium, and chloride generally increased with increasing proportion of Grand Calumet River sediment, and hydrogen sulfide was low (<0.003 μg/L) in all of the porewater samples (Supplemental Data, Table S2). Concentrations of ammonia were elevated (>9 mg N/L) in all of the sediment treatments except for the sand control. The range of hardness, alkalinity, conductivity, pH, sulfate, sodium, potassium, and chloride tended to be lower in West Bearskin Lake sediment dilutions than in sand dilutions. The relative percent deviation of a duplicate sample analysis was <15% for the water quality characteristics of the porewater.

**Toxicity testing**

Conditions for conducting the toxicity tests are summarized in the Supplemental Data, Table S1. Methods for conducting the sediment toxicity tests with *H. azteca* and *C. dilutus* were based on procedures outlined by ASTM International [1] and the USEPA [2]. The method for conducting the sediment toxicity tests with *L. siliquoidea* were adapted from water-only testing methods outlined in ASTM International [7] and Wang et al. [9–11].

Addition of overlying water to exposure beakers was started 24 h before the start of the exposures with an automated water delivery system that provided approximately 2 water volume additions per day [18]. Ambient laboratory light (widespectrum fluorescent lights) was approximately 500 lux at the water surface of the exposure beakers with 16:8-h light:dark photoperiod. The overlying water was prepared by mixing well water with deionized water to a water hardness of approximately 100 mg/L as CaCO3, alkalinity of 85 mg/L as CaCO3, conductivity of approximately 250 μS/cm at 25°C, pH of 8.2, and dissolved organic carbon of approximately 0.5 mg/L. Mean water quality values were similar among sediment samples within a test species or life stages of species tested, ranging from hardness 102 mg/L to 130 mg/L as CaCO3, alkalinity of 74 mg/L to 100 mg/L as CaCO3, conductivity of 250 μS/cm to 302 μS/cm at 25°C, dissolved oxygen of 4.5 mg/L to 7.7 mg/L, pH of 7.5 to 8.2, and ammonia of 0.03 mg N/L to 1.3 mg N/L (Supplemental Data, Table S3).

The *H. azteca* and *C. dilutus* were obtained from cultures at the CERC Laboratory [25]. Exposures were started with approximately 7-d-old *H. azteca* (mean starting size, 0.02 mg dry wt/individual) and 7-d-old *C. dilutus* larvae (mean starting size, 0.028 mg ash-free dry wt [AFDW]/individual for West Bearskin Lake sediment treatments and 0.058 mg AFDW/individual for sand treatments; Supplemental Data, Table S1). Because of its small size, the weight of the <1-h-old *C. dilutus* was not determined at the start of the exposures. Approximately 6-wk-old *L. siliquoidea* (mean starting size 0.20 mg dry wt/individual) used to start the exposures were obtained from organisms propagated at Missouri State University (Springfield, MO, USA) and cultured at the CERC Laboratory [9–11,17].

Test organisms were acclimated to test water and temperature for a minimum of 24 h before the start of the exposures. Ten test organisms were impartially transferred into each of 4 replicate exposure beakers at the beginning of the exposures. For the first 8 h after addition of test organisms, there was no delivery of water to allow the <1-h-old *C. dilutus* larvae to better settle into sediment [1,2].
Toxicity endpoints evaluated included: treatment 1a: *H. azteca* 10-d survival, weight, and biomass; treatment 1b: *H. azteca* 28-d survival, weight, and biomass (i.e., total weight of surviving organisms in each replicate); treatment 2a: *C. dilutus* 20-d survival, AFDW, and biomass started with <1-h-old larvae; treatment 2b: *C. dilutus* 48-d adult emergence started with <1-h-old larvae; treatment 3a: *C. dilutus* 13-d survival, AFDW, biomass started with 7-d-old larvae; treatment 3b: *C. dilutus* 48-d adult emergence started with 7-d-old larvae; and treatment 4: *L. siliquoidea* 28-d survival, weight, and biomass started with approximately 6-wk post-transformation juveniles.

Treatments 1 and 2 were conducted following traditional chronic sediment toxicity testing methods for *H. azteca* or *C. dilutus* [1,2]. Treatments 3a and 3b represent modifications to the chronic *C. dilutus* sediment toxicity testing method that has been used by the CERC Laboratory for several years because of difficulty with control survival in tests conducted by the CERC Laboratory and by other laboratories in exposures started with <24-h-old *C. dilutus*. Treatment 4 represents methods adapted from ASTM International [1] and the USEPA [2] for sediment toxicity testing with invertebrates and adapted from ASTM [7] for water-only mussel toxicity testing.

*Hyalella azteca* were fed a ramped amount of yeast-cerophyll-trout chow (YCT) suspension (1.8 mg solids) once food has been found to improve growth of test day 4 to end of exposures, 1.5 mL daily. A ramped diet of *H. azteca* fi <20-d survival, AFDW, and biomass started with surviving organisms in each replicate; treatment 2a: *A. azteca* each replicate was estimated as the sum of individual amphipod weight measurement. The biomass of surviving *L. siliquoidea* of survival (foot movement within a 5-min observation period) containing approximately 20 mL of water for the determination recovered and transferred into a 50-mL glass exposure beaker connected to a computer and a microscope. Surviving *C. dilutus* fi and remaining sediment from the sieve into a system to allow more time for the food to be consumed by test organisms.

At the end of the sediment toxicity tests, test organisms were isolated from each exposure beaker by rinsing the sediment through a US #50 sieve (0.3-mm opening) and then rinsing the test organisms and remaining sediment from the sieve into a glass tray. Live *H. azteca* and *C. dilutus* in each replicate were counted. Surviving *H. azteca* in each replicate were preserved in 8% sugar formalin for subsequent length measurements [18]. Lengths of *H. azteca* at the end of test were measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface using a digitizing system with image micrometer software (Image Caliper, Resolution Technology) connected to a computer and a microscope. Surviving *C. dilutus* in each replicate were placed in a preweighed aluminum pan for the measurement of AFDW (after ashing at ~550 °C for 2 h). The *L. siliquoidea* in each replicate exposure beaker were recovered and transferred into a 50-mL glass exposure beaker containing approximately 20 mL of water for the determination of survival (foot movement within a 5-min observation period) using a dissecting microscope. Surviving *L. siliquoidea* in each replicate were preserved in 8% formalin for subsequent dry weight measurement. The biomass of surviving *H. azteca* from each replicate was estimated as the sum of individual amphipod weights calculated from the empirical relationship [16,17,26]

$$\text{weight (mg)} = (0.177 \times \text{length (mm)}) - 0.0292$$

Dry weight of surviving *L. siliquoidea* in each replicate was determined after the mussels were dried for 24 h at 60 °C. The dry weight was measured to the nearest 0.001 mg with a microbalance (Model MX5, Mettler Toledo).

**Data analysis**

The lowest-observed-effect concentration (LOEC) for all endpoints was determined with TOXSTAT software (Ver 3.5; Western EcoSystems Technology) by one-way analysis of variance (ANOVA) with mean comparison made by one-tailed Dunnett’s test (or Bonferroni t-test when the number of replicates was not the same for all concentrations [27]). If data were not normally distributed or did not have equal variances, Steel’s many one rank test or Wilcoxon rank sum test with Bonferroni adjustment was used for the determinations of the LOEC. The level of statistical significance was set at α = 0.05. The 20% effect concentrations (EC20s) were estimated where applicable by normal probability distribution analysis for survival data and by nonlinear regression analysis (logistic equation model) for dry weight, biomass, and emergence data using the Toxicity Relationship Analysis Program (Ver 1.21 [28]). The exposure concentrations were log-transformed, and the response of each replicate was used for the EC20 calculations.

Sediment toxicity benchmarks, which were derived from empirically based probable effect concentrations (PECs) for metals, PAHs or PCBs, were used to assess relationships between sediment chemistry and toxicity. The PECs are effect-based sediment quality guidelines established as concentrations of individual chemicals above which adverse effects in sediments are expected to frequently occur in field-collected sediments [29]. Mean quotients based on PECs were calculated to provide an overall measure of chemical contamination and to support an evaluation of the combined effects of multiple contaminants in sediments [21]. Individual PEC quotients (PECQs) were calculated for each of 5 metals (SEM concentrations of Cd, Cu, Pb, Ni, and Zn) and 13 PAHs (acenaphthene, acenaphthylenne, anthracene, benzo[a]anthracene, benzo[a]pyrene, chrysene, dibenzo[a,h]anthracene, fluoranthene, fluorene, 2-methylanthanthene, naphthalene, phenanthrene, and pyrene) in each sediment sample by dividing the dry-weight concentration of the chemical by the PEC for that chemical [29]. To weight the contribution of metals or PAHs equally in the evaluation of sediment chemistry and toxicity, an average PECQ for metals or for total PAHs was calculated for each sample [21].

Mechanistically based ΣESBTUs also were estimated for 34 parent and alkylated PAHs from the 16 measured parent PAHs [20]. To adjust for alkylated PAHs not quantified by analysis for only 16 parent PAHs, an uncertainty factor of 50% was applied to the ΣESBTU that was calculated from the data on the 16 parent PAH concentrations [20]. The ΣESBTU approach was developed to account for the biological availability of non-ionic organic compounds in different sediments and incorporates select biological effects concentrations in porewater (i.e., final chronic values [20]).

**RESULTS AND DISCUSSION**

**Amphipod *Hyalella azteca* toxicity testing**

Mean control survival at the end of the 10-d and 28-d exposures conducted with *H. azteca* was ≥95% in West Bearskin Lake sediment and in sand (Supplemental Data, Table S4). Mean control dry weight at the end of the 28-d exposures was 0.62 mg/individual in West Bearskin Lake
sediment (a proportional increase of 31×) and was 0.38 mg/individual in sand (a proportional increase of 19×). Hence, mean control survival of *H. azteca* in the 10-d and 28-d exposures met test acceptability criteria for survival (80%) and weight (an increase in weight during the exposures [1,2]; Supplemental Data, Table S4). However, dry weight of *H. azteca* in the sand control tended to be lower than control dry weight in the West Bearskin Lake sediment control (no absolute test acceptability criteria are provided by ASTM International [1] and the USEPA [2] for *H. azteca* control ending dry wt in [1,2]). Subsequent studies have determined that alternate diets either of ramped diatoms (*Thalassiosira weissflogii*) and ramped Tetramin or of YCT and ramped Tetramin result in improved performance of *H. azteca* in the sand control relative to sediment controls (D.R. Mount, unpublished data).

Dilutions of Grand Calumet River sediment with sand (low TOC) were toxic to *H. azteca* in 10-d and 28-d exposures, resulting in nearly complete mortality at every dilution (Table 2; Supplemental Data, Table S4). Dilutions of Grand Calumet River sediment with West Bearskin Lake sediment (high TOC) also were toxic to *H. azteca*. Survival of *H. azteca* in the 10-d and 28-d exposures was reduced significantly only in 50% Grand Calumet River sediment dilution with West Bearskin Lake sediment; however, the magnitude of response was greater in 28-d exposures (Figure 1A). More consistent dose responses and lower EC20s in West Bearskin Lake sediment dilutions for weight or biomass were observed in the 28-d exposures than in the 10-d exposures conducted with *H. azteca* (e.g., biomass EC20 of 40% Grand Calumet River sediment for 10-d exposure and biomass EC20 of 13% Grand Calumet River sediment for the 28-d exposure; Table 2 and Figure 1B).

*Midge C. dilutus* toxicity testing

Mean control survival of larvae was ≥96% and mean control AFDW was ≥1.68 mg/individual at the end of the 13-d 28-d exposures. Error bars represent standard deviations.

Table 2. Lowest-observed effect concentrations (LOECs) and 20% effect concentrations (EC20) in exposures conducted with East Branch Grand Calumet River (GCR) sediment diluted with West Bearskin control sediment (WB) or diluted with sand in exposures conducted with amphipods (*Hyalella azteca*), midges (*Chironomus dilutus*), and mussels (*Lampsilis siliquoidea*).

| Dilution sediment          | GCR effect concentration (%) | Survival (%) | Weight (mg/individual) | Biomass (mg) | Emergence (%) |
|---------------------------|------------------------------|--------------|------------------------|--------------|---------------|
| *H. azteca* 10-d exposures |                              |              |                        |              |               |
| WB                        | LOEC                        | 50           | 12.5                   | 50           | —             |
|                           | EC20                        | 37 (32–42)   | 45 (no CI)             | 40 (no CI)   | —             |
| Sand                      | LOEC                        | 6.25         | —                      | 6.25         | <6.25         |
|                           | EC20                        | <6.25        | —                      | <6.25        | —             |
| *H. azteca* 28-d exposures|                              |              |                        |              |               |
| WB                        | LOEC                        | 50           | 12.5                   | 12.5         | —             |
|                           | EC20                        | 26 (no CI)   | 22 (17–29)             | 13 (7–25)    | —             |
| Sand                      | LOEC                        | 6.25         | —                      | 6.25         | —             |
|                           | EC20                        | <6.25        | —                      | <6.25        | —             |
| *C. dilutus* 20-d exposures (larval survival, weight, biomass) or 48-d exposures (emergence) started with 1-h-old larvae |                              |              |                        |              |               |
| WB                        | LOEC                        | 12.5         | >50                    | 12.5         | >50           |
|                           | EC20                        | <6.25        | <50                    | <6.25        | <6.25         |
| Sand                      | LOEC                        | 6.25         | —                      | 6.25         | <6.25         |
|                           | EC20                        | <6.25        | —                      | <6.25        | <6.25         |
| *C. dilutus* 13-d exposures (larval survival, weight, biomass) or 48-d exposures (emergence) started with 7-d-old larvae |                              |              |                        |              |               |
| WB                        | LOEC                        | 50           | 25                     | 9.4 (2–50)   | 29 (11–74)    |
|                           | EC20                        | >50          | 18 (7–46)              | 25 (10–46)   | —             |
| Sand                      | LOEC                        | 6.25         | 6.25                   | 6.25         | —             |
|                           | EC20                        | <6.25        | <6.25                  | <6.25        | <6.25         |
| *L. siliquoidea* 28-d exposures |                              |              |                        |              |               |
| WB                        | LOEC                        | >50          | 6.25                   | —            | —             |
|                           | EC20                        | >50          | 6.25                   | —            | —             |
| Sand                      | LOEC                        | 25           | 12.5                   | 12.5         | —             |
|                           | EC20                        | >12.5        | <12.5                  | <12.5        | <12.5         |

*Not applicable or not determined.

b95% confidence intervals (CI) in parenthesis.

![Figure 1](image-url) Mean survival (A) and biomass (B) relative to the control of the amphipod (*Hyalella azteca*) exposed to East Branch Grand Calumet River (GCR) sediment diluted with West Bearskin (WB) sediment in 10-d and 28-d exposures. Error bars represent standard deviations.
exposures started with 7-d-old *C. dilutus* (Supplemental Data, Table S5). Mean control emergence of adult *C. dilutus* in the exposures started with 7-d-old larvae was ≥88%. Emergence of adult *C. dilutus* was determined through test day 41 in sand exposures and through test day 48 in West Bearskin Lake exposures. Hence, mean control survival, weight, and emergence of *C. dilutus* in exposures started with 7-d-old larvae met or exceeded test acceptability criteria for survival (70%), weight (0.48 mg/individual), and emergence (50%) [1,2].

Mean control survival was 71% in West Bearskin Lake exposure and was 92% in sand at the end of the 20-d exposures started with <1-h-old *C. dilutus* larvae (Supplemental Data, Table S5). Mean control AFDM was ≥2.49 mg/individual at the end of the 20-d exposures started with <1-h-old larvae. Mean control emergence of adult *C. dilutus* was 48% in West Bearskin Lake sediment and was 82% in sand in the exposures started with <1-h-old larvae. Emergence of adult *C. dilutus* was determined through test day 41 in sand exposures and through test day 48 in West Bearskin Lake exposures. Hence, mean control survival and weight of *C. dilutus* in exposures started with <1-h-old larvae met the test acceptability criteria for survival and weight [1,2] (Supplemental Data, Table S5). In West Bearskin Lake control sediment, however, replicate variability was high for survival (100%, 100%, 67%, 17%). Replicate variability for emergence in West Bearskin Lake control sediment was also high (100%, 66%, 17%, 8%), with mean emergence of 48% (slightly below the test acceptability criterion of 50% recommended by ASTM International [1] or the USEPA [2]).

Dilutions of Grand Calumet River sediment with sand (low TOC) were toxic to *C. dilutus* in 20-d exposures started with <1-h-old larvae or in 13-d exposures started with 7-d-old larvae (Table 2; Supplemental Data, Table S5). The lowest sand dilution of 6.25% Grand Calumet River sediment resulted in 100% mortality in exposures started with <1-h-old larvae and significantly reduced survival, AFDM, biomass, and percent emergence in exposures started with 7-d-old larvae (EC20s < 6.25% Grand Calumet River sediment for both life stages; Table 2).

Dilutions of Grand Calumet River sediment with West Bearskin Lake sediment (high TOC) also were toxic to *C. dilutus* in exposures started with either life stage (Table 2; Supplemental Data Table S5), though less toxic in the West Bearskin Lake sediment dilutions than in sand dilutions. In the exposures started with <1-h-old larvae, ≥12.5% Grand Calumet River sediment dilution with West Bearskin Lake sediment significantly reduced survival and biomass (EC20s < 6.25% Grand Calumet River sediment dilution with West Bearskin Lake sediment). In the exposures started with 7-d-old larvae, ≥25% Grand Calumet River sediment dilution with West Bearskin Lake sediment significantly reduced AFDM and biomass, and the 50% Grand Calumet River sediment dilutions with West Bearskin Lake sediment significantly reduced percent emergence (EC20 for biomass of 9.4% Grand Calumet River sediment and EC20 for emergence of 29% Grand Calumet River sediment). Median emergence time of *C. dilutus* was not consistently delayed with exposure to the dilutions of Grand Calumet River sediment with either West Bearskin Lake sediment or with sand (data not shown).

In exposures to West Bearskin Lake sediment dilutions, survival of *C. dilutus* was a more sensitive endpoint in the 20-d exposures started with <1-h-old larvae (Figure 2A) than was survival or biomass in the 13-d exposures started with 7-d-old larvae (Figure 2B). As mentioned, however, control replicate variability was high in West Bearskin Lake control sediment in exposures started with <1-h-old larvae (Figure 2A; Supplemental Data, Table S5). In the exposures to West Bearskin Lake sediment dilutions started with 7-d-old larvae, the magnitude of the response was similar for larval biomass and percent adult emergence (Figure 2B). Hence, percent emergence did not provide additional information on the toxicity of the sediment dilutions beyond effects on larvae biomass observed by test day 13 of the exposure.

**Mussel *Lampsilis siliquoidea* toxicity testing**

Mean control survival was 93% in West Bearskin Lake sediment and was 100% in sand at the end of the 28-d exposures conducted with *L. siliquoidea*. Mean control dry weight was 0.62 mg/individual in West Bearskin Lake sediment (a proportional increase of 3.1×) and was 0.34 mg/individual in sand (a proportional increase of 1.7×). Mean control survival of *L. siliquoidea* met the test acceptability criterion of 80% survival for mussels in water-only exposures (Supplemental Data, Table S6) [7]. Variability tended to be relatively high for starting size of *L. siliquoidea* (e.g., starting length of 1.67 mm/individual, standard deviation 0.32 mm/individual) and was relatively high within some of the treatments at the end of the exposures (e.g., 25% Grand Calumet River sediment dilution with West Bearskin Lake sediment; Supplemental Data, Table S6) 

![Figure 2](image-url)
This variability likely resulted from having a few larger mussels within some replicates, which could substantially inflate inter-replicate variability. Improvements to the culturing procedures of mussels at the CERC Laboratory have helped reduce variability in starting weights of mussels. Ideally, about twice the number of mussels needed to start an exposure should be produced to avoid using larger or smaller mussels to start an exposure. Increasing the number of replicates tested from 4 to 8 also may help reduce variability in weight of mussels at the end of an exposure.

Dilutions of Grand Calumet River sediment with sand (low TOC) or with West Bearskin Lake sediment (high TOC) were toxic to *L. siliquoidea* (Table 2; Supplemental Data, Table S6). Survival of *L. siliquoidea* in sand dilutions was reduced significantly at ≥25% Grand Calumet River sediment (EC20 of 16% Grand Calumet River sediment); however, survival in West Bearskin Lake sediment dilutions was not reduced in any of the exposures (Figure 3A and Table 2; Supplemental Data, Table S6). In contrast, weight or biomass of *L. siliquoidea* was reduced significantly with exposure to ≥6.25% Grand Calumet River sediment dilutions with West Bearskin Lake sediment or to ≥12.5% Grand Calumet River sediment dilutions with sand (Figure 3B and Table 2; Supplemental Data, Table S6). Survival, weight, and biomass of *L. siliquoidea* were affected more severely in sand dilutions than in West Bearskin Lake sediment dilutions. However, EC20s could not be calculated for weight or biomass because of the limited dose response in exposures between 6.25% and 50% Grand Calumet River sediment dilutions with either West Bearskin Lake sediment or with sand (Figure 3B and Table 2).

Relative species and endpoint sensitivity

Relative species sensitivity was primarily compared for the survival and biomass endpoints for the 28-d exposures with *H. azteca* and *L. siliquoidea* and the 13-d exposures started with 7-d-old *C. dilutus*. These endpoints and exposures were selected because other endpoints (e.g., weight or midge emergence endpoints) or exposures (e.g., *H. azteca* 10-d exposure) tended to be less responsive than were the survival or biomass endpoints illustrated in Figure 4 for sand dilutions and in Figure 5 for West Bearskin Lake sediment dilutions. The 20-d exposures started with <1-h-old *C. dilutus* were not included in Figure 4 and in Figure 5 because of poor control response in West Bearskin Lake sediment dilutions (Figure 2A).

Survival of *L. siliquoidea* in sand dilutions was a less sensitive endpoint than was survival of *C. dilutus* or *H. azteca* (Figure 4A). Similarly, biomass of *L. siliquoidea* in sand dilutions tended to be a less sensitive endpoint relative to biomass of *C. dilutus* or *H. azteca* (Figure 4B).
siliquoidea and C. dilutus in West Bearskin Lake sediment dilutions was a less sensitive endpoint relative to survival of H. azteca (Figure 5A). In contrast, biomass of L. siliquoidea in West Bearskin Lake sediment dilutions tended to be a more sensitive endpoint relative to biomass of C. dilutus or H. azteca (Figure 5B). Revisions are planned to the ASTM methods [1,7] and to USEPA method [2] to further describe biomass as a toxicity endpoint that can be used to integrate combined effects of a treatment on survival or weight of test organisms.

Previous water or sediment toxicity testing conducted with juvenile freshwater mussels has demonstrated that growth (reported as dry wt or length) tends to be a more sensitive endpoint relative to survival [9,11,15–17,30]. Mussels may be avoiding exposure to contaminants by valve closure, but this avoidance may come at a cost of reduced feeding and reduced growth. For example, there were multiple treatments in sand dilutions or West Bearskin Lake sediment dilutions where survival was above 80%, yet weight exhibited little or no increase during the 28-d exposures (Supplemental Data, Table 5S). Avoidance behavior might also have contributed to variable dose response relationships observed in some of the previous 28-d water or sediment toxicity tests conducted at the CERC Laboratory with juvenile mussels [9–11].

The test acceptability criterion for 28-d control survival of juvenile mussels in water-only toxicity tests has been established at ≥80% [7]. The CERC Laboratory has adapted these water-only methods to develop a method for conducting 28-d whole-sediment toxicity tests with juvenile mussels (Supplemental Data, Table S1). Supplemental Data, Table S7 provides a summary of historic control performance in sediment exposures conducted by the CERC Laboratory with a variety of species of freshwater mussels. Results of these studies indicate that 28-d control survival averaged 91% (n = 34), and 91% of these studies exhibited control survival ≥80%. Average increase in 28-d control length was 1.6 fold (n = 28) and average increase in 28-d weight was 4.2 fold (n = 25). In the 3 instances in which 28-d control survival was <80% in a study, it is likely that the batch of mussels used for that study was of lower quality (e.g., mussels in cultures used to generate those mussels in that sediment study also did poorly; N. Wang, unpublished data). Recent studies conducted by the CERC Laboratory have demonstrated improved growth of mussels in 28-d to 84-d water or sediment exposures when the amount of algal food was increased to 3 mL twice daily from the 2 mL twice daily in the present study (N. Wang, unpublished data). Data from these historic and ongoing studies will be used to draft a planned ASTM International method for conducting whole-sediment toxicity tests with juvenile mussels (e.g., as a revision to the ASTM International sediment method [1] or as a revision to ASTM International mussel water-only method [7]).

Survival of C. dilutus in exposures started with <1-h-old larvae was typically the most sensitive endpoint across all species and endpoints in exposures of Grand Calumet River sediment diluted with sand or diluted with West Bearskin Lake sediment (Table 2). Younger C. dilutus larvae tend to be more sensitive than older larvae in acute 2-d to 4-d water toxicity tests [1,2]. In contrast, similar sensitivity was observed in exposures started with <1-h-old C. dilutus and with 7-d-old C. dilutus in toxicity testing conducted with PCB-contaminated sediments [22]. However, control survival of <1-h-old larvae in West Bearskin Lake sediment in the present study was only 71% with high control variability (Figure 2A). Previous studies have demonstrated variable and inconsistent control survival in sediment or water exposures started with <1-h-old larvae of C. dilutus [22,31,32]. Studies are ongoing to determine if improved performance of C. dilutus can be achieved in exposures started with <1-h-old larvae relative to exposures started with about 4-d-old to 7-d-old larvae. Results of these studies will be used to revise guidance in the ASTM method [1] and in the USEPA method [2] for conducting chronic sediment or water toxicity tests with C. dilutus.

Adult emergence and larval biomass of C. dilutus in exposures started with 7-d-old larvae exhibited similar toxicity patterns with exposure to Grand Calumet River sediment diluted with sand or diluted with West Bearskin Lake sediment (Figure 2B). Hence, either reduced larval survival or reduced larval weight may result in subsequent reductions in emergence of adult C. dilutus. In contrast, emergence was more than 13-fold more sensitive than larval biomass in exposures to PCB-contaminated sediments [22] and was more than 4-fold more sensitive in water exposures to the fungicide Azoxystrobin (J.L. Kunz, unpublished data). Similarly, adult emergence was a more sensitive endpoint relative to larval biomass in exposures to metal-contaminated sediments from the Upper Columbia River in eastern Washington State (J.M. Besser, US Geological Survey, Columbia, MO, USA, unpublished data). Ingersoll et al. [22] reported that reproductive endpoints (e.g., egg number and hatching of larvae) were not affected by exposure to PCB-contaminated sediments. Additional studies and evaluations are
needed to determine which endpoints provide the most useful indicators of the chronic toxicity of contaminants to midges; the utility of indicators may vary by chemical class or mode of chemical action. Measuring reproductive endpoints of *C. dilutus* beyond emergence requires testing of numerous replicates (e.g., 16 replicates/treatment [1,2]), compared with only 8 replicates tested in the present study to determine larval biomass and adult emergence.

Weight and biomass of *H. azteca* in the 28-d exposures were 2-fold to 3-fold more sensitive relative to the 10-d exposures in the Grand Calumet River sediment diluted with West Bearskin Lake sediment (based on EC20s in Table 2 and Figure 1B). Previous studies with *H. azteca* have demonstrated that longer-term exposures may or may not be more sensitive than short-term exposures [18,31]. However, the amount of food provided to *H. azteca* in previous 28-d exposures was likely low (e.g., 1 mL of YCT based on guidance provided in ASTM International [1] and USEPA [2], relative to an increased amount YCT provided in the present study [Supplemental Data, Table S1]), which resulted in increased 28-d control weight relative to historic studies (Supplemental Data, Table S4). Subsequent intra- and inter-laboratory studies have demonstrated even better control performance of *H. azteca* in 42-d water or sediment reproduction tests when provided a diet either of ramped diatoms and ramped Tetramin, or of YCT and ramped Tetramin (C.G. Ingersoll and D.R. Mount, unpublished data). These alternate ramped diets for conducting sediment or water toxicity tests with *H. azteca* will be included in revisions being made to the ASTM International sediment toxicity method [1] and to the USEPA sediment toxicity method [2].

**Relationships between toxicity and chemistry**

The 100% Grand Calumet River sediment sample was highly toxic to *H. azteca* and *C. dilutus* (100% mortality) in a previous 10-d exposure and contained a complex mixture of metals and organic contaminants that may have contributed to the toxicity (Table 1). The EC20s or LOECs in the present study for the most sensitive endpoints for each test organism ranged from <6.25% to 13% Grand Calumet River in dilutions with West Bearskin Lake sediment and were often <6.25% Grand Calumet River in dilutions with sand (Table 2). For the 6.25% and 12.5% Grand Calumet River sediment dilutions with sand or with West Bearskin Lake sediment, the overall mean PECQs (based on PECQs for total PCBs, total PAHs, and a mean metal PECQ) ranged from 0.35 to 0.81. This range of overall mean PECQs was similar to the threshold for toxicity for *H. azteca* or midges reported for mean PECQs, ranging from approximately 0.5 to 1.0 in a freshwater toxicity database [21] and was similar to a toxic mean PECQ of approximately 0.63 reported in a previous 28-d toxicity test conducted with *H. azteca* exposed to a Grand Calumet River sediment diluted with West Bearskin Lake sediment [31].

The mean metal PECQs in the 6.25% and 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment or with sand ranged from 0.61 to 1.43 (Table 1), which are similar to the threshold for toxicity reported for mean metals PECQs ranging from approximately 0.5 to 1.0 [21]. However, concentrations of metals in the Grand Calumet River sediment diluted with West Bearskin Lake sediment would not be expected to be toxic due to metals because an estimated excess of AVS relative to SEM [19] in all of the West Bearskin Lake sediment dilutions tested would likely reduce the bioavailability of metals (i.e., the 100% Grand Calumet River sediment sample and West Bearskin Lake sediment both likely had concentrations of AVS >32.2 μmole/g; Table 1). In contrast, concentrations of bioavailable metals in the Grand Calumet River sediment diluted with sand might have increased with increasing dilutions with sand. Diluting Grand Calumet River sediment with sand with no AVS might have resulted in oxidation of AVS in the Grand Calumet River sediment, particularly in the 12.5% and 6.25% Grand Calumet River sediment dilutions (e.g., testing sand coated with Grand Calumet River particles, with the potential to oxidize AVS and release dissolved metals into porewater). Hence, toxicity attributable to metals cannot be ruled out in the sand dilutions.

Concentrations of total ammonia in the porewater at the start of the exposures were elevated in all of the sediment dilutions tested except for the sand control (>9 mg N/L; Supplemental Data, Table S2); however, the estimated concentrations of un-ionized ammonia were relatively low in the 6.25% and 12.5% Grand Calumet River sediment dilutions (<0.58 mg/L in sand dilutions and <0.027 mg/L in West Bearskin Lake sediment dilutions). Concentrations of ammonia in porewater of sediments likely decreased during the exposure with the daily renewal of overlying water (C.G. Ingersoll, unpublished data). Kemble et al. [26] reported effect concentrations of un-ionized ammonia nitrogen of >1.5 mg N/L for *H. azteca* in 28-d exposures and 0.94 mg N/L for *C. dilutus* in 10-d exposures. According to Wang et al. [11], the un-ionized ammonia effect concentrations ranged from 0.013 to 0.057 mg/L for *L. siliquoidea*. Hence, ammonia did not likely cause the observed toxicity to *H. azteca* or *C. dilutus* in the 6.25% or 12.5% Grand Calumet River sediment dilutions. However, toxicity to *L. siliquoidea* in the sand dilutions because of ammonia cannot be ruled out.

The estimated concentrations of total PCBs or total PAHs in the 6.25% to 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment or with sand were at or below various sediment toxicity thresholds (Table 1). For example, the PECQs for total PCBs in the 6.25% and 12.5% Grand Calumet River sediment dilutions ranged from 0.26 to 0.59, which were at or below a range of PECQ sediment toxicity threshold for total PCBs of 0.5 to 1.0 [21] and were at or below other PCB sediment toxicity thresholds [22,33]. Similarly, the PECQs for total PAHs in the 6.25% and 12.5% Grand Calumet River sediment dilutions ranged from 0.19 to 0.40, which were below PECQ sediment toxicity thresholds for total PAHs of 0.5 to 1.0 [21]. The ΣESBTU for PAHs in the 100% Grand Calumet River sediment sample was 2.46 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon, which is below an estimated *H. azteca* 28-d toxicity threshold of approximately 17 μmole/g organic carbon. The estimated concentrations of oil in the 6.25% to 12.5% Grand Calumet River sediment dilutions with sand dilutions and in the 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment were at or above sediment toxicity thresholds (Table 1). The toxicity of oil in sediment was more strongly correlated on a volume basis relative to a dry-weight basis (i.e., aliphatic hydrocarbons expressed as solvent extractable organic matter [SEOM]; Mount et al., unpublished data). Therefore, concentrations of oil range organics measured in the present study were expressed as a volume basis by multiplying the dry weight concentration (mg oil range organics/g) by the fraction solids (g dry wt/g wet wt) and by wet density (g/mL) in each sediment dilution (Table 1). A 10-d EC50 was estimated to be 1.31 g SEOM/L for biomass of *C. dilutus* and 1.36 g SEOM/L for biomass of *H. azteca* (Mount, unpublished data).
et al., unpublished data). Concentrations of oil range organics were higher in sand dilutions (e.g., 3.9 g/L in the 6.25% Grand Calumet River sediment dilution and 7.2 g/L in the 12.5% Grand Calumet River sediment dilution) relative to West Bearskin Lake sediment dilutions (0.52 g/L in the 6.25% Grand Calumet River sediment dilution and 1.1 g/L in the 12.5% Grand Calumet River sediment dilution; Table 1). Hence, concentration of oil likely contributed to the toxicity observed in the 6.25% and 12.5% Grand Calumet River sediment dilutions with sand and in the 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment. However, there is some uncertainty in comparing oil measured as SEOM with oil measured as oil range organics.

In summary, the toxicity may have been caused by ammonia alone (to mussels) or metals alone in the 6.25% or 12.5% Grand Calumet River sediment dilutions with sand. Concentrations of oil alone may have caused the toxicity observed in the 6.25% or 12.5% Grand Calumet River sediment dilutions with sand and in the 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment. The metals, PCBs, or PAHs alone in the 6.25% and 12.5% Grand Calumet River sediment dilutions with West Bearskin Lake sediment likely did not cause the toxicity, but these 3 groups of chemicals may have contributed to the toxicity in the 6.25% and 12.5% Grand Calumet River sediment dilutions with sand or with West Bearskin Lake sediment. Biomass of the test organisms tended to be the most responsive endpoint. While exposures started with 1-h-old larvae of C. dilutus tended to be the most sensitive life stage and species tested, control variability confounded the interpretation of the exposures started with younger larvae relative to exposures started with 7-d-old larvae of C. dilutus.

SUPPLEMENTAL DATA

Tables S1–S7.

Figure S1 (281 KB PDF).

Acknowledgment—We would like to thank the members of the Columbia Environmental Research Center Toxicology Branch for technical support. We thank T. Augspurger and 2 anonymous reviewers for providing comments on the manuscript. Funding for the present study was provided in part by US Environmental Protection Agency Region 5.

Disclaimer—Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government. The US Environmental Protection Agency has not formally reviewed this publication, and the views expressed herein may not reflect the views of the US Environmental Protection Agency.

Data Availability—Data beyond the data not summarized in the primary manuscript or in the supplemental data can be accessed by contacting Chris Ingersoll (cingersoll@usgs.gov).

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