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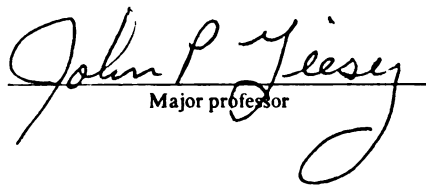
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**LABORATORY AND FIELD ANALYSES OF THE POTENTIAL TOXICITY OF
POLYDIMETHYLSILOXANE (SILICONE) TO
BENTHIC MACROINVERTEBRATES**

By

Kevin S. Henry

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ABSTRACT

LABORATORY AND FIELD ANALYSES OF THE POTENTIAL TOXICITY OF POLYDIMETHYLSILOXANE TO BENTHIC MACROINVERTEBRATES

By

Kevin S. Henry

Polydimethylsiloxane (PDMS) is widely used in a number of industrial processes and consumer products that result in down-the-drain disposal. Negligible volatility and water solubility and a slow degradation rate suggest that PDMS may accumulate in sediments. Sediment bioassays with the amphipod *Hyaletta azteca* and with larvae of the midge *Chironomus tentans* were used to assess the potential for toxicity of PDMS-amended sediments to benthic invertebrates in short-term and whole life cycle exposures. Endpoints for short-term tests included survival and growth, while life cycle assays considered survival, growth, reproduction, and emergence. Potential effects of PDMS on benthic communities were investigated *in situ* in plastic trays containing sediments that had been defaunated and spiked with PDMS to concentrations of 1000 ppm (nominal d.w.). Artificial substrate trays were placed at a depth of 10 m in Lake Orta, Italy, a lake which has been historically contaminated with ammonia and heavy metals. Trays were collected after 3 mo and invertebrates sieved and identified to genus or species, when possible. Short-term and life cycle laboratory exposures indicated that environmentally relevant concentrations of PDMS will not significantly decrease survival, growth, or reproduction. *In situ*, artificial substrate exposures exhibited a high degree of variability among treatments, and suggested possible impacts on oligochaetes. However, this observation was not substantiated in laboratory exposures with the oligochaete *Lumbriculus variegatus*.

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**Dedicated to my Mother, Jean Henry,
for encouraging my interest in every reasonable pursuit.**

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INTRODUCTION

Polydimethylsiloxane (PDMS) fluids are synthetic polymers consisting of an alternating silicon-oxygen backbone with methyl side groups (Figure 1). PDMS is used in a variety of industrial and consumer applications, including heat transfer fluids, antifoams, cosmetics, waxes and polishes (Hamelink 1992). Many of these uses result in down-the-drain disposal. The most common linear siloxanes are insoluble in water and extremely hydrophobic, therefore, siloxanes become associated with sludge solids upon reaching sewage conduits and waste water treatment plants (WWTPs, Watts et al. 1995; Fendinger et al. 1997).

Approximately 3% of the PDMS delivered to WWTPs has been estimated to be released to surface waters in the United States (Fendinger et al. 1997). This represents an total annual loading of approximately 294,000 kg/y (Fendinger et al. 1997). Upon entering surface waters, the majority of the suspended sludge and associated PDMS will eventually deposit into bottom sediments. The small fraction of PDMS that is not sorbed to suspended particulates in the effluent would partition to suspended solids in the river or lake water and also eventually become deposited into the bottom sediments (Fendinger et al. 1997).

Degradation of PDMS is believed to occur by hydrolysis to yield monomeric dimethylsilanediol (DMSD, Watts et al. 1995), though this reaction is inversely related to soil moisture content (Lehmann et al. 1998). In one study, a year of incubation in aerated sediments resulted in hydrolysis of 5-10% of PDMS present to DMSD (Carpenter et al. 1996). The rate of PDMS degradation was less in sterile sediments, which suggests that this process may be related to sediment microflora (Carpenter et al. 1996). The mobility

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of PDMS in aquatic systems will be primarily limited to resuspension and deposition of sediment particles with which it is associated. Concentrations of PDMS in sediments decrease with depth, and are not detectable at depths which correspond to periods before the production and use of silicone fluids (Batley and Hayes 1991; Pellenberg and Tevault 1986). While PDMS tends to accumulate in sediments, concentrations can decrease when inputs are stopped, by dilution and degradation (Carpenter et al. 1996). PDMS concentrations in sediments downstream from WWTPs have been observed to range from 0.9 to 78 mg/kg, dw at sites receiving point source inputs. However, 90% of the sediments tested contained less than 26 mg PDMS/kg, dw (Powell et al. 1996). The biota exposed to PDMS would primarily be benthic invertebrates intimately associated with these sediments.

The reported toxicity of PDMS to benthic invertebrates is low. In part, this nominal level of toxicity is due to the fact that there is little bioaccumulation (Table 1). It has been suggested that this low bioaccumulation potential is due to the large molecular weight of PDMS molecules, which are believed to be too large to cross biological membranes (Frye 1983). While PDMS has been found to be nontoxic in short-term studies, little information was available on the probable chronic toxicity of PDMS to sediment dwelling benthic invertebrates. PDMS is surface active so it was hypothesized that large concentrations in sediments could change the physical characteristics of the sediments. For instance, it has been reported that PDMS can reduce the bioavailability of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) from sediments (Kukkonen and Landrum 1995). Because a number of benthic invertebrates extract organic compounds from sediments, either as an energy source or in the form of fat

soluble vitamins, it was postulated that PDMS might affect the availability of the organic compounds by acting as a cosolvent. Finally, because PDMS may affect the texture of sediments with respect to their viscosity or degree of consolidation, there was concern that PDMS-contamination of sediments could change the suitability of the sediments for benthic invertebrates. If the degree of consolidation of the sediment is altered by PDMS, bioturbation and the production of burrows may be adversely impacted.

The objectives of this study were to determine if sediment-bound PDMS will affect the survival, growth, or reproduction of benthic invertebrates. This was investigated by use of field studies of mixed populations of naturally occurring invertebrates and controlled laboratory bioassays with several different species. Test methodologies used included standardized short-term bioassays as well as multiple life-stage protocols which are currently under development by the USEPA and USFWS.

MATERIALS AND METHODS

Test Organisms and Sediment

Three species of invertebrates were used in laboratory exposures: an amphipod (*Hyalella azteca*, Crustacea) a midge (*Chironomus tentans*, Insecta) and an aquatic worm (*Lumbriculus variegatus*, Oligochaeta). All of these species demonstrate characteristics of ideal test species (Giesy and Hoke 1989). Standard test methods using these organisms exist, and survival and growth are the primary endpoints (USEPA 1994; ASTM 1995). Therefore, these organisms were used as surrogates for insects, crustacea and oligochaetes in natural communities. The three organisms selected represent a range of possible tolerances and life histories.

All three species were reared and maintained according to methods adapted from Environment Canada (1997a, b), Kubitz et al. (1996), and USEPA (1994). Briefly, *H. azteca* were cultured at $23 \pm 1^\circ\text{C}$ in 1 L plastic breeding chambers kept in an incubator. Nylon bolting cloth was used as a substratum. Amphipods were fed daily a suspension of yeast, cereal leaves, and trout chow (i.e. YCT), as well as the green alga *Selanastrum capricornutum*. Partial water replacements were performed two times per week. *C. tentans* and *L. variegatus* were cultured in 19 L aerated glass aquaria to which shredded and presoaked unbleached paper towels were added as a substratum. *C. tentans* were fed a suspension of blended fish food flakes (Tetramin), and *L. variegatus* were fed a suspension of ground trout chow daily. Water for all cultures consisted of a mixture containing 30% well water and 70% Barnstead E-Pure (Dubuque, IA), resulting in a final hardness of approximately 90 mg CaCO_3/L and a final alkalinity of approximately 100 mg CaCO_3/L .

Test sediment for all lab studies except those conducted with “low organic carbon” sediment was collected from a small pond in Williamston, Michigan, which receives no regular surface water inputs. Low organic carbon sediments from Lake Michigan were obtained from Peter Landrum at the NOAA/Great Lakes Environmental Research Laboratory, Ann Arbor, Michigan. Sediment from Williamston contained 4% organic carbon (OC), while that from Lake Michigan contained 0.5% OC. Sediments were stored in high-density polyethylene buckets in the dark at 4°C until use.

PDMS Fluid

Polydimethylsiloxane fluid with a viscosity of 350 cs was obtained from Dow Corning Corporation (Midland, MI). The molecular weight of this fluid was 17970 and

the log K_{ow} was approximately 10. Vapor pressure and water solubility are below detection limits for PDMS of this viscosity.

Sediment Dosing and Analyses

Sediment spiking methodologies were designed to be as environmentally realistic as possible. PDMS-amended treatments were prepared by spinning sludge, PDMS and water in a round-bottomed flask on a Rotavap in order to mix these components. This spiked sludge was then stirred into homogenized sediments using a drill-mounted stainless steel propeller. The sediment/sludge mixture was shaken on a platform shaker, then permitted to equilibrate undisturbed for at least 7 d at 4°C before it was homogenized again for use in bioassays.

Concentrations of PDMS in the sediments were determined on subsamples. Dosed wet sediment samples were taken in duplicate or triplicate for PDMS analyses. PDMS concentrations were determined by multiple extraction of sediment subsamples with tetrahydrofuran (THF), followed by ICP analyses of the extracts (Fendinger et al. 1997b; Appendix 1).

Short-Term Laboratory Exposures

Short-term sediment bioassays were conducted using procedures similar to those developed by the U.S. Environmental Protection Agency (USEPA 1994) and Environment Canada (1997a, b). Standard operating procedures are attached (Appendix 2 and 3). Bioassays with *C. tentans* were conducted on cohorts reared from eggs deposited on the same date. Larvae of uniform age (13 d post-oviposition) and uniform size were selected for use in bioassays. Bioassays with *H. azteca* were performed with 7-8 day old organisms. Tests with *L. variegatus* were initiated with adults.

Bioassays were conducted in a Benoit system (Benoit et al. 1982). Exposure chambers (4-16 per replicate) were prepared from 300 mL high-form borosilicate glass beakers with two 17 mm holes, covered with 100 μ m stainless steel screen, to allow water circulation. Bioassay chambers were filled with 100 mL of test sediment and 175 mL of overlying water and placed into the exposure aquarium one day before the start of a test. One volume of dilution water was added to each aquarium before the start of a bioassay and the exposure chamber water was maintained at $23\pm 1^{\circ}\text{C}$ over the course of a test. Test animals were added to the chambers at random to produce a total of ten animals per chamber. Each group of replicates was placed in a 19 L glass aquarium, into which water replacements were added by gravity from a polyethylene head tank. The water replacement rate was two volumes per day.

C. tentans and *H. azteca* were fed suspensions of Tetramin or YCT, respectively, each day. *L. variegatus* were not fed during a test. Feeding and water renewals continued in this fashion until termination of the bioassay after 10 d. Dissolved oxygen (DO) was measured in two replicates of each treatment daily before water replacement. If persistent reductions in DO (<40% saturation) were detected, the number of water exchanges per day was increased. If necessary, feeding rates were also reduced temporarily in all treatments. Composite samples of overlying water for water quality analysis were collected on test days 0 and 10.

At the end of a bioassay, exposure chambers were removed from the aquaria and the contents of each chamber were sieved through a 425 μ m (*H. azteca* and *L. variegatus*) or 710 μ m (*C. tentans*) sieve and rinsed with culture water. The number of survivors in each chamber was recorded and the weight of surviving *C. tentans* and *L. variegatus*

individuals determined to calculate growth. *C. tentans* individuals were pooled by replicate in pre-ashed aluminum weigh boats and dried for 24 h at 60°C to determine dry weight. Ash-free dry weight (AFDW) was also estimated to reduce the bias introduced by gut contents (Sibley et al. 1997). To find AFDW, the weigh pans containing the dry larvae were ashed at 550°C for 2h. The pans with the ashed larvae were then re-weighed and the tissue mass of the larvae determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan. *L. variegatus* were pooled by replicate and wet biomass determined. Oligochaetes were not dried because they were later used in supplemental experiments which required wet biomass and which are not described here.

Whole Life Cycle Assays

Whole life cycle assays with *C. tentans* and *H. azteca* were conducted based on draft protocols developed by the USEPA and during a series of round-robin tests in which our laboratory participated. Standard operating procedures are attached (Appendix 4 and 5). Minimum data quality objectives for survival, growth and reproduction have been suggested (USEPA 1999). These values are given below where available.

C. tentans Whole Life Cycle Assay

The life cycle test for *C. tentans* was modified from Benoit et al. (1997) and USEPA (1999). Bioassay chambers were prepared as described above under short-term laboratory exposures. Specialized equipment specific to the midge whole life stage assay is described in the standard operating procedure below (Appendix 4). Briefly, adult midges are placed into a 500 mL Erhlenmeyer flask for mating. The following day, 6 - 8 of the larger C-shaped egg masses are transferred to a glass crystallizing dish containing

culture water and incubated at $23 \pm 1^{\circ}\text{C}$. *C. tentans* eggs incubated at this temperature begin hatching after 48 h and the larvae start leaving the egg mass 24 h after first hatch (72 h total). To start a test (day 0 of test), newly hatched larvae are collected with a Pasteur pipette from the bottom of the incubation dish with the aid of a dissecting microscope. Twelve test organisms were pipetted directly into the overlying water of each exposure chamber. Each midge test beaker was fed 1.0 mL of a 4 g/L Tetramin suspension daily starting on day -1.

A total of 12 beakers per replicate were used in the test, and an additional 4 beakers were prepared and midge larvae introduced on day 10 for production of auxiliary males to be used in reproduction. These beakers are necessary because peak female emergence may occur up to 1 week later than that of male *C. tentans* (Sadler 1935). The additional replicates ensure a sufficient supply of reproductively viable males for mating with females from the experimental treatments during the latter stages of the emergence period.

On day 20 of the test, the sediment from four randomly selected beakers per treatment was sieved through a 40 mesh (425 μm) screen to recover larvae for growth and survival determinations. Growth was measured as ash-free dry weight. Data quality objectives for mean control survival and for mean AFDW of 70% and 0.48 mg/control individual have been suggested (USEPA 1999). After 20 d, emergence traps (screened lids) were placed over the remaining eight reproductive replicates. Emergence traps for the auxiliary male beakers are added at the corresponding 20 d time interval.

Male/female emergence and adult mortality in the test chambers were recorded daily. Adults were collected daily from each individual test chamber and transferred to

the corresponding reproduction/oviposit (R/O) chamber for breeding. Each R/O unit was checked daily for dead adults and for egg cases. Dead flies were removed. For each emerged female, at least one male derived from the corresponding reproductive replicate, another replicate of that treatment, or from the auxiliary male beakers was transferred into the R/O unit. A draft quality control value for percent emergence of 60% has been suggested (USEPA 1999).

Egg masses from the R/O chambers were transferred to incubation dishes and the number of eggs determined either by estimation using a ring count method or by direct counting after digestion in an acid solution. The ring count method involves multiplying the mean number of eggs contained in 5 rings of the egg mass by the number of rings present. The acid method is used when the integrity of an egg mass precludes estimation by the ring method, such as when the mass is convoluted or distorted. Under these circumstances, egg masses were soaked overnight in sulfuric acid. The acid dissolves the gelatinous matrix surrounding the eggs and permits counting of individual eggs. The ring count method is preferable as it is faster and permits determination of egg hatching success. Each intact egg mass counted by the ring method was incubated in culture water and incubated at 23°C until hatching was complete, which normally takes between 2 and 6 d. Hatching success was determined by subtracting the number of unhatched eggs remaining from the number of eggs originally estimated for that egg mass. Draft quality assurance objectives of 900 eggs per female and 90% hatch have been suggested by the USEPA (1999).

The physicochemical properties of the sediments determine the length of this test. In nontoxic sediments, the test typically requires 40 to 50 d for completion. However,

test duration increases in the presence of stressors which act to reduce growth and delay emergence. The test was terminated when there was no emergence from the control treatments for 7 d. At test termination, all beakers were sieved through a #40 mesh screen (425 μ m) to recover remaining larvae, pupae, or pupal cases. The endpoints for the test were thus 20 d growth and survival, emergence, reproduction in the number of eggs per female, and percent hatch.

H. azteca Whole Life Cycle Assay

H. azteca used in the whole life cycle assay were 7 to 8 days old. To start a test, amphipods of known age were collected with a Pasteur pipette from the bottom of a crystallizing dish with the aid of a dissecting microscope. A total of 12 test beakers were set up for each treatment and test organisms pipetted directly into the overlying water of the exposure beakers. Each beaker received a daily addition of 1.0 mL of YCT starting on day -1, which is the day preceding test initiation.

Endpoints monitored included 28d survival and growth of *H. azteca* and 35 d and 42 d survival, growth, and reproduction (number of young/female). On day 28 of the test, 4 of the replicate beakers/sediment were separated with a #40 mesh sieve (425- μ m mesh) to remove surviving *H. azteca*. Immobile organisms isolated from the sediment surface or from sieved material were considered dead. Dry weights were found at 20 d for surviving amphipods in these 4 replicates. Control survival and dry weight data quality objectives of 80% and 0.15 mg/individual have been suggested (USEPA 1999).

After 28 d, the remaining 8 of the original 12 beakers/sediment were also sieved and the surviving *H. azteca* in each beaker placed into 300 mL beakers containing 250

mL of overlying water and a 5-cm x 5-cm piece of Nitex® screen. Each water-only beaker received 1.0 mL of YCT stock solution and two volume additions of water daily.

Reproduction of amphipods was measured after 35 d and 42 d in the water-only beakers by removing and counting the adults and young in each beaker. On day 35, the adults were returned to the same water-only beakers. Adult amphipods surviving at 42 d were preserved in an 8% sugar formalin solution. The number of adult males was enumerated based on the size of the second gnathopod, which is enlarged in male *H. azteca*. The number of surviving females was used to determine the number of young/female/beaker produced between 28 d and 42 d. A data quality objective of 2 young/female has been suggested (USEPA 1999). Dry weight for these surviving *H. azteca* were also be measured.

In Situ Bioassays

In situ bioassays were conducted in Lake Orta, Italy. This alpine lake has received heavy metal- and ammonium-containing industrial discharges since 1926 (Mosello and Calderoni 1990). The effects of this contamination on lake biota were almost immediate and the lake was relatively devoid of fauna by the end of 1927. Since then, effluent treatment and liming of the lake have resulted in a partial recovery (Mosello and Calderoni 1990). Lake Orta sediments were collected and a portion of the sediments amended with PDMS-treated sludge using the spiking method described above. Sediments of three PDMS treatment levels (10, 100, and 1000 mg PDMS/kg, dw nominal) were generated, in addition to a sludge blank (0 mg PDMS/kg, dw nominal), and a control blank which contained neither sludge nor PDMS. The treated and untreated sediments were placed into 14 L plastic trays and positioned on the lake bed in

10 m of water with a method adapted from Hare et al. (1995a, b). One tray of each sediment concentration was recovered after three months, and sieved using a plankton net to insure retention of early instar midges. The invertebrates present were then identified to the greatest degree possible, generally genus or species.

Statistics

Mean treatment effects of PDMS were examined statistically by a combination of parametric and nonparametric methods. Percent data were arcsin square root transformed before testing. All data were assessed for homogeneity of variance using Bartlett's test and for normality by Shapiro-Wilk's test. If the data met these assumptions, parametric statistics were used. If one or more assumptions were not met, nonparametric statistics were used. Data were evaluated by ANOVA followed by Dunnett's test for pairwise comparison between each treatment and the control means. Nonparametric tests were performed using the same tests on rank transformed data. Type I (α) error for all tests was set to 0.05.

RESULTS AND DISCUSSION

Short Term Laboratory Exposures

Survival of *C. tentans* and *H. azteca* were not affected during 10 d exposures to PDMS-spiked sediments. Survival of *C. tentans* larvae exposed in two separate experiments to concentrations of up to 1502 or 2590 mg PDMS/kg, dw was not different than that of individuals in control sediments (Figure 2a, 3a). Survival of *H. azteca* was not significantly less in sediments containing up to 1900 mg PDMS/kg dw than that of controls (Figure 4).

Growth of *C. tentans* was significantly less in an initial exposure to PDMS-spiked sediments, though this result was not replicated when the exposure was repeated. The dry weight of *C. tentans* exposed to treatments of 161 or 1562 mg PDMS/kg, dw was significantly less than that of those grown on control sediments (Figure 2b). The ash-free dry weight of these individuals was significantly less than that of the controls only in the 1562 mg PDMS/kg, dw (Figure 2c). This experiment was repeated and the number of replicates was doubled to 16 per treatment. In the second exposure there were no statistically significant differences in dry weight or ash-free dry weight between any of the silicone-spiked sediments and control sediment (Figure 3b, c). The experiment was repeated a third time for confirmation, and again there were no statistically significant reductions in growth in PDMS-spiked sediments (data not shown).

Organic carbon (OC) content of sediment did not affect responses of *C. tentans* or *H. azteca* to PDMS. Virtually all particles in aquatic systems carry an organic coating that can potentially sorb chemical contaminants (Stumm 1992), and the partitioning of hydrophobic compounds to this coating is often great (Karickhoff and Morris 1985; Swackhammer and Skoglund 1991). Sediment OC content has been reported to influence the bioavailability and toxicity of some compounds (e.g. Adams 1987; Swartz et al. 1990). Therefore, the potential exists that organic carbon content of sediments may affect the degree of sorption and perhaps the toxicity of PDMS. Kukkonen and Landrum (1995) found no reduction in growth or survival of *L. variegatus* in Lake Michigan sediments of 0.5% OC content treated with 150 mg PDMS/kg dw. In the present study, no differences in growth or survival of *C. tentans* or *H. azteca* were observed in 10 d exposures to Lake Michigan sediments collected from the same site as those in the

Kukkonen and Landrum study (1995) and spiked with PDMS up to a concentration of approximately 1200 mg PDMS/kg dw (Figure 5).

Whole Life Cycle Assays

While short-term tests may be used to study the potential for acute toxicity, they may not be able to predict subtle effects from long-term exposures at the community level (Benoit et al. 1997; Ingersoll et al. 1998). In order to better evaluate the certainty that PDMS is not causing adverse effects in aquatic environments, assessments of the potential for chronic, sublethal impacts rather than acute toxicity were made by use of whole life cycle studies with *C. tentans* and *H. azteca*.

Survival, growth, emergence and reproduction of *C. tentans* in PDMS-treated sediments at concentrations up to 2600 mg PDMS/kg, dw were not significantly different than from that of *C. tentans* on untreated sediment. Suggested data quality objectives of 70% survival and 0.48 mg/kg AFDW were achieved in the controls, and values of these parameters at all PDMS treatments also exceeded these levels (Figure 6). Percent emergence of adult *C. tentans* was not significantly affected by PDMS-treated sediments, although neither control nor treatment means exceeded the 60% data quality objective suggested by the USEPA (Figure 7a). During recent round-robin testing, only 43% of the labs participating met this emergence criterion (USEPA 1999), indicating that the 60% level may be greater than what should typically be expected. This 60% emergence value and the other QA parameters for whole life cycles assays were based on testing in a single laboratory (Sibley et al. 1996; Sibley et al. 1997; Benoit et al. 1997), and may not reflect likely results in all laboratories (USEPA 1999). There were no significant effects of PDMS on the number of eggs produced per female, and mean production for all

treatments was greater than the suggested egg production value of 900 eggs for controls (Figure 7b). Percent hatch of the eggs was not significantly different in any of the spiked sediments from the control, and all treatments exceeded the 90% suggested data quality level (Figure 7c). PDMS in sediments did not influence either egg production or percent hatch, which suggests that reproduction of *C. tentans* will not be adversely impacted by sediment concentrations of up to 2600 mg PDMS/kg, dw.

Survival, growth, and reproduction of *H. azteca* in whole life cycle assays were not significantly reduced in PDMS-spiked sediments relative to control sediments up to concentrations as great as 994 mg PDMS/kg,dw. Survival of amphipods at 28, 35 and 42 d in PDMS-treated sediments was not significantly different from that in control sediments, although the percent survival in the control treatment decreased from 87% after 28 d to 77% and 63% after 35 and 42 d, respectively (Figure 8). While the data quality objective of 80% survival after 28 days was achieved, these values were somewhat less than the mean survivals of 90 (28 d), 89 (35 d), and 86% (42 d) in the control sediment from West Bearskin Lake during the round-robin assay (USEPA 1999). These contrasts may be due to differences in physicochemical parameters of the sediments, such as grain size, which make the Williamston Pond sediment less ideal for amphipod colonization. Growth of *H. azteca* was not significantly different between PDMS-spiked and control sediments after 28 or 42 d (Figure 9a, b), although variability was great in the least PDMS treatment after 28 d (CV = 43% in 9 mg PDMS/kg, dw). This is likely due to the few replicates for this endpoint (N = 4), and could be improved with increased replication. The control data quality objective of 0.15 mg/individual after 28 d was achieved in all treatments. Reproduction in all treatments exceeded the

suggested control data quality value of 2 young per female and there was no difference between any individual PDMS treatment and the control (Figure 9c). The suggested minimum data quality objective for reproduction in control treatments of 2 young per female was exceeded in all treatments. However, the reproduction endpoint was more variable than either growth or survival. Coefficients of variation were 64, 90, 71, and 64% in the control, 9, 104, and 994 mg PDMS/kg, dw treatments, respectively. This great variability suggests that this may not be an appropriate measure of contaminant effects, or increased replication may be needed to improve statistical resolution of this endpoint.

In Situ Bioassays

While acute and sublethal endpoints can be measured in the laboratory with some degree of accuracy and can provide unique data on the potential toxicity of sediments, the ecological consequences of exposure to contaminants may more realistically be evaluated at higher levels of organization (Underwood and Peterson 1988; Clements and Kiffney 1994). Field studies with artificial substrates were designed to simulate a realistic exposure scenario; that of sewage sludge outfall into a lake ecosystem.

Measured PDMS concentrations for PDMS-spiked artificial substrate treatments were 13.4, 75.7, and 798 mg PDMS/kg dw upon collection. The control blank, which was treated with neither sludge nor PDMS, and the sludge blank contained 3.0 and 2.3 mg PDMS/kg, dw respectively, which indicates some level of background contamination in these sediments. This study site lies within a bay that contains a public beach and it is possible that the background PDMS levels are an artifact of suntan lotion deposited by bathers.

Almost all of the macroinvertebrates present in the trays after in situ incubation were chironomids or oligochaetes. The abundance of chironomids in the three PDMS-spiked treatments was within 30% of control blank values, while the number of individuals (392 larvae) in the sludge blank was only about 50% of the control blank level of 773 animals (Figure 10a). The percent of predaceous midges in the treatments ranged from 27 to 41% (Figure 10b). Oligochaete abundances in the sludge blank and at 13.4 and 75.7 mg PDMS/kg, dw were 58, 76 and 71% the number in the control blank, respectively (Figure 10a). The total number of oligochaetes in the greatest PDMS treatment (798 mg PDMS/kg,dw) was only 8% that of the untreated control blank and 14% that of the sludge-only control (Figure 10a).

The most prominent difference observed among treatments was the decrease in oligochaete abundance in the presence of 798 mg/kg PDMS/kg, dw, the greatest concentration tested (Figure 10a). Several explanations were considered, including the presence of a greater number of predators in this treatment, or the possibility that PDMS may be adversely impacting oligochaetes. The proportion of predaceous midges at 798 mg PDMS/kg, dw was less than that of the control blank and the sludge blank, and similar to that of the 13 and 76 mg PDMS/kg, dw treatments, thus suggesting that predators are not the source of the effect.

The observation of apparent PDMS-related effects on oligochaetes under field conditions was the impetus for laboratory exposures with the oligochaete *Lumbriculus variegatus*. Short-term laboratory bioassays were conducted which indicated no potential toxicity from PDMS in sediments based on survival and growth (Figure 11). This lack of observed effects is similar to the results of other studies with PDMS-spiked sediments

and aquatic worms, including *L. variegatus* (Aubert et al. 1985; Guillemaut-Drai et al. 1988; Craig and Caunter 1990; Kukkonen and Landrum 1995). It is possible that the variability among treatments in the in situ assays was due to the patchy structure of natural invertebrate communities, and we hope to understand this better upon sorting further samples.

In summary, the present study corroborates and expands upon previous research (Table 1) which has indicated minimal toxicity of PDMS to sediment-dwelling organisms. PDMS in sediments did not affect growth, survival, or reproduction of *C. tentans* or *H. azteca* in laboratory tests at concentrations that were at least 20 fold greater than those which occur in the environment. Reproduction of *H. azteca* was too variable to observe clear trends within treatments. The suggestion that silicone may decrease oligochaete abundance in field studies was contradicted by the published literature and could not be substantiated in our own lab studies. Results of this study indicate that current concentrations of PDMS fluid in the aquatic environment will not induce significant effects on benthic invertebrate assemblages.

Table 1. Effects of polydimethylsiloxane on benthic invertebrates.

Test Organism	Maximum Concentration Tested	Endpoint and Result	Reference
Polychaete <i>Nereis diversicolor</i>	10,000 mg/kg in sediment	No reduction in growth or survival to 28 d though burrowing activity was affected, perhaps due to pretreatment of sediments rather than to PDMS	Craig and Caunter 1990
Polychaete <i>Nereis diversicolor</i> ; Mussels <i>Mytilus edulis</i> & <i>Mytilus galloprovincialis</i> ; Crab (Crustacean) <i>Carcinus maenas</i>	10,000 mg/L in water	Low toxicity and bioaccumulation/biomagnification potential in marine trophic chains	Aubert et al. 1985; Guillénaut et al. 1987
Oligochaete <i>Lumbriculus variegatus</i>	150 mg/kg in sediment	No toxicity or bioaccumulation; rapid depuration of PDMS. PDMS in sediments reduced the bioaccumulation of Benzo[a]pyrene	Kukkonen and Landrum 1995

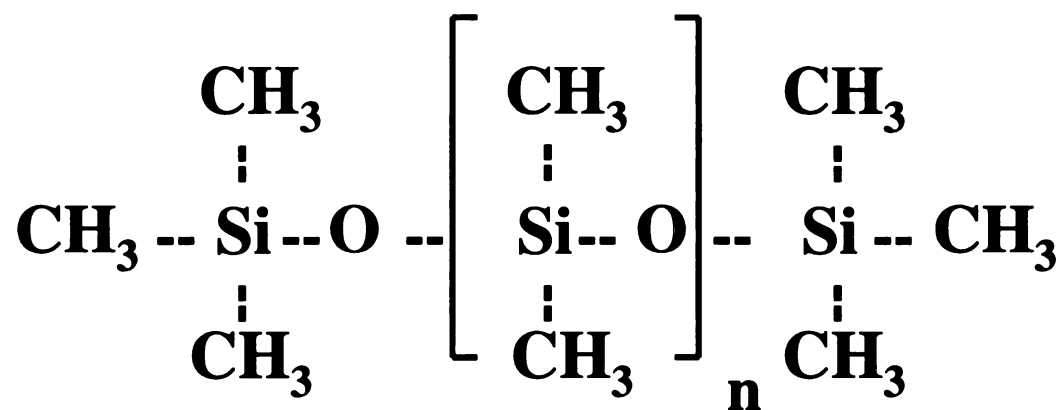
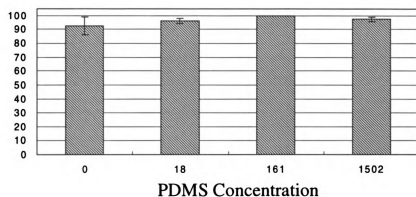


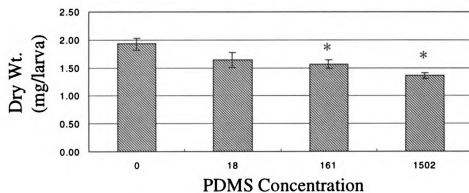
Figure 1. Generic structure of Polydimethylsiloxane (PDMS).
 n can range from 10 to >10,000 (Stark et al. 1982).

Figure 2. Responses in *Chironomus tentans* bioassays with PDMS-spiked sediments: Trial 1 of 2. Means, with standard error (N = 16). * indicates statistically significant difference at $\alpha = 0.05$ using Dunnett's test for differences between each treatment and a control. (a) growth measured as dry weight per individual; (b) growth measured as ash-free dry weight per individual.

(2a) *Chironomus tentans* Assay 1: Survival



(2b) *Chironomus tentans* Assay 1: Dry Weight



(2c) *Chironomus tentans* Assay 1: Ash-Free Dry Weight

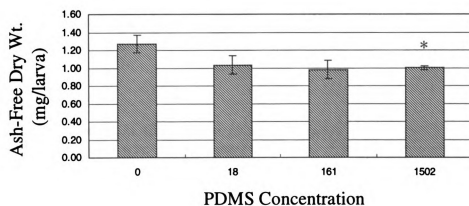
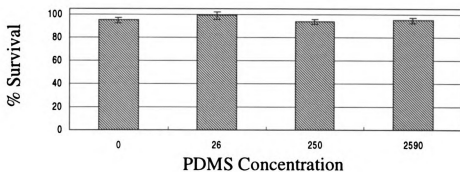
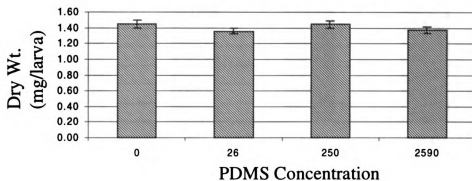


Figure 3. Responses in *Chironomus tentans* bioassays with PDMS-spiked sediments: Trial 2 of 2. Means, with standard error (N = 16). * indicates statistically significant difference at $\alpha = 0.05$ using Dunnett's test for differences between each treatment and a control. (a) growth measured as dry weight per individual; (b) growth measured as ash-free dry weight per individual.

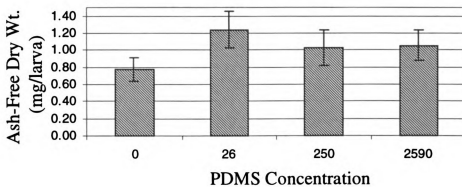
(3a) *Chironomus tentans* Assay 2: Survival



(3b) *Chironomus tentans* Assay 2: Dry Weight



(3c) *Chironomus tentans* Assay 2: Ash-Free Dry Weight



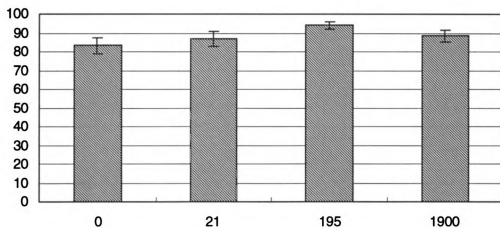
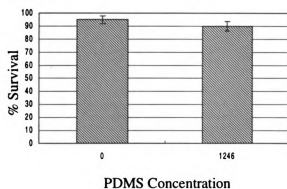


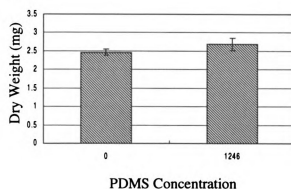
Figure 4. Survival in *Hyalella azteca* bioassay with PDMS-spiked sediments. Means, with standard error (N = 12).

Figure 5. Responses of *Chironomus tentans* and *Hyaella azteca* with PDMS-spiked sediments of low organic carbon content (0.5% OC). Means, with standard error. (a) percent survival of *Chironomus tentans* (N = 16). (b) growth of *Chironomus tentans* measured as dry weight per individual (N = 16). (c) percent survival of *Hyaella azteca* (N = 12). (d) growth of *Hyaella azteca* measured as dry weight per individual (N = 12).

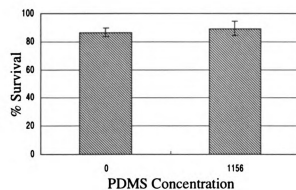
(5a)



(5b)



(5c)



(5d)

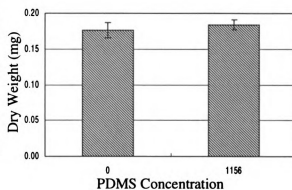
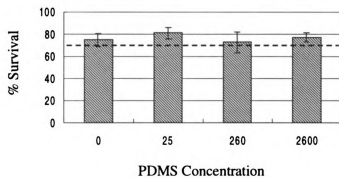


Figure 6. Responses in *Chironomus tentans* whole-life cycle bioassays with PDMS-spiked sediments. Dashed lines indicate suggested USEPA data quality objectives. (a) percent survival at 20 d (N = 4). (b) Ash-free dry weight at 20 d (N = 4).

(6a) *C. tentans* % survival at 20 d in whole-life cycle assay.



(6b) *C. tentans* growth at 20 d in whole-life cycle assay.

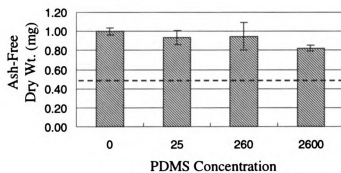
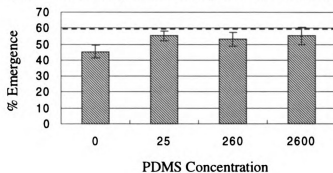
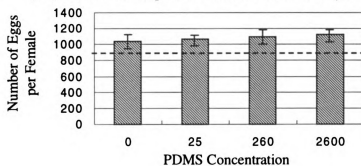


Figure 7. Responses in *Chironomus tentans* whole-life cycle bioassays with PDMS-spiked sediments. Dashed lines indicate suggested USEPA data quality objectives. (a) *C. tentans* percent emergence (N = 8). (b) reproduction in number of eggs/female. (c) percent of eggs hatching.

(7a) *C. tentans* percent emergence in whole-life cycle assay.



(7b) *C. tentans* reproduction in whole-life cycle assay.



(7c) *C. tentans* percent egg hatch in whole-life cycle assay.

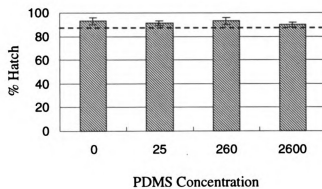
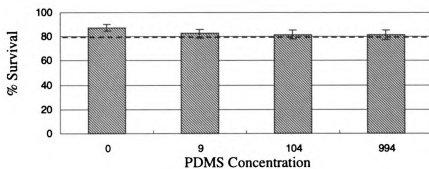
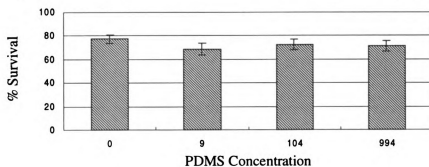


Figure 8. Responses in *Hyaella azteca* whole-life cycle bioassays with PDMS-spiked sediments. Dashed lines (when present) indicate suggested USEPA data quality objectives. (a) percent survival at 28 d when animals are isolated from sediments and placed into water-only chambers (N = 12). (b) Percent survival at 35 d (N= 8). (c) Percent survival at 42 d (test termination, N = 8).

(8a) *H. azteca* percent survival at 28 d in whole-life cycle assay.



(8b) *H. azteca* percent survival at 35 d in whole-life cycle assay.



(8c) *H. azteca* percent survival at 42 d in whole-life cycle assay.

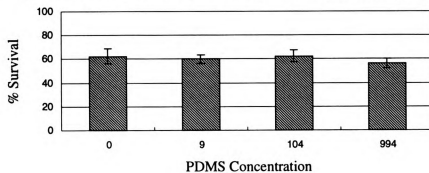
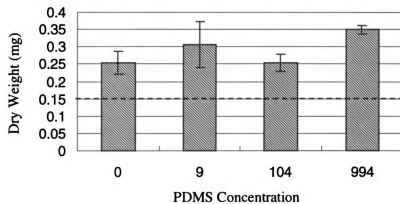
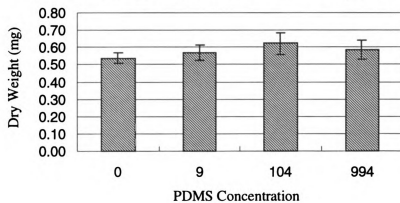


Figure 9. Responses in *Hyaella azteca* whole-life cycle bioassays with PDMS-spiked sediments. Dashed lines (when present) indicate suggested USEPA data quality objectives. (a) growth at 28 d when animals are isolated from sediments and placed into water-only chambers (N = 4). (b) growth at 42 d (test termination, N = 8). (c) reproduction at 42 d evaluated in number of young produced per female.

(9a) *H. azteca* growth at 28 d in whole-life cycle assay.



(9b) *H. azteca* growth at 42 d in whole-life cycle assay.



(9c) *H. azteca* reproduction.

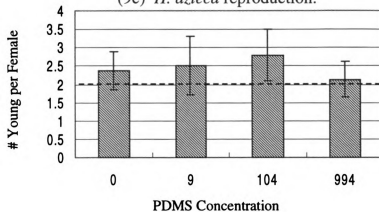
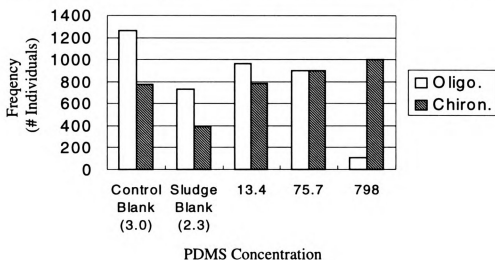


Figure 10. Results of *in situ* study with silicone-spiked sediments. (a) histogram of oligochaetes and chironomids in artificial substrates. (b) percent of chironomid predators in artificial substrates.

(10a) Histogram of oligochaetes and chironomids in artificial substrates.



(10b) Percent of chironomid predators in artificial substrates.

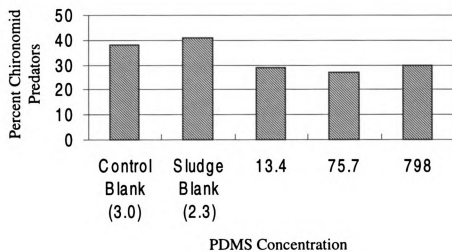
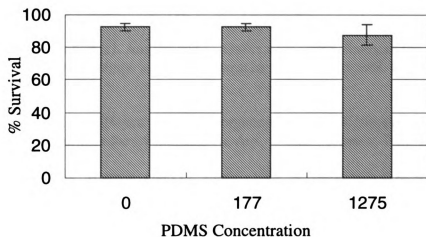
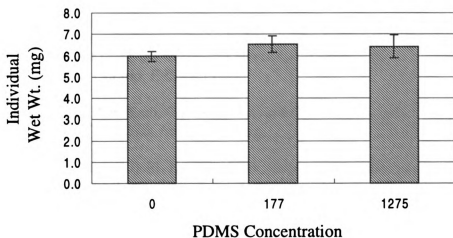


Figure 11. Responses in *Lumbriculus variegatus* bioassays with PDMS-spiked sediments. Means, with standard error (N = 4). (a) percent survival. (b) growth measured as mean wet weight per individual.

(11a) *Lumbriculus variegatus* survival in PDMS-spiked sediments.



(11b) *Lumbriculus variegatus* growth in PDMS-spiked sediments.



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APPENDICES

APPENDIX 1: STANDARD PROCEDURE FOR POLYDIMETHYLSILOXANE (PDMS) EXTRACTION FROM SEDIMENT

Aquatic Toxicology Laboratory Standard Operating Procedure

Department of Zoology

Michigan State University

Title:

Polydimethylsiloxane (PDMS) Solvent Extraction from Sediment for Analysis by GPC-ICP

Description of Method:

This protocol has been adapted from SOP NE209_02.GLP provided by Northeast Analytical (NEA) to Dow Corning Corporation. Contact at NEA is Inga C. Hotaling (518) 346-4592. To avoid concentration and degradation of the sample, materials should be sealed in plastic and stored at 4°C. This method involves a multiple tetrahydrofuran (THF) extraction of test sediments to isolate the PDMS present. Extracts are evaporated to near dryness and shipped offsite for analysis by ICP.

Hazards and Precautions:

Care must be exercised when using solvents. THF is moderately toxic and very volatile and flammable. Extractions should be done in a well-ventilated hood.

Materials:

HPLC grade Tetrahydrofuran (THF) Available from MSU Stores.

Teflon (preferable) or Nalgene wash bottle for THF storage and delivery

Clean beakers

Volumetric pipette (10 mL)

Wrist-action or platform shaker.

Fume hood

Laboratory centrifuge

35 mL Kimax heavy duty centrifuge tubes (VWR#: 21023-252) For triplicate extractions, 6 tubes per sample will be required; 4 tubes per sample for duplicate extractions

Teflon-lined caps for Kimax tubes

Aluminum weigh pans for sediment dry:wet weight ratios

Drying oven

Pasteur pipets (short stem) for solvent transfer

Procedure:

Dry:Wet Weight Ratio

- 1) Place 5 ± 0.3 g of wet, homogenized sediment onto a pre-dried and pre-weighed aluminum weigh pan. Record actual weight of sediment.
- 2) Dry sample for at least 24 h at 60°C.
- 3) Weigh pan + sediment and record weight.
- 4) Calculate dry weight as the difference between the pan + dry sediment weight and the pan weight.
- 5) Calculate dry:wet weight ratio as dry sediment weight / wet sediment weight.

Sediment Sample Preparation

- 1) Rinse all glassware and pipets with THF before use. Dispose of THF in hazardous waste container.

- 2) Remove sediment sample from refrigerator and allow sample to equilibrate to ambient temperature. Homogenize sample within the sample container using a stainless steel spoon.
- 3) Weigh approximately 10.0 g of sediment into a centrifuge tube and record sample weight to 0.01 g. Samples should be done at least in duplicate. If sample contains excessive water, tubes may be sealed and centrifuged for 3 minutes at high speed to isolate solids. Water may then be pipetted off and discarded.
- 4) Add 10 mL of THF to each centrifuge tube, seal with a Teflon-lined cap and shake for 10 min on laboratory shaker.
- 5) Centrifuge sample on high speed for 3 min to isolate solids. Transfer THF into another clean and THF-rinsed centrifuge tube. Blow down extract under a low flow of nitrogen.
- 6) Repeat steps B4-B5 two more times, combining the extracts and blowing down to dryness after the third extraction step.
- 7) Add 0.5 mL of water to prevent PDMS degradation and ship sealed and labeled tubes to Dow Corning for analysis as soon as possible.

Extraction Blank Preparation

Repeat steps 4-7 with an empty centrifuge tube (no soil).

Reporting concentrations of PDMS results in soil, sediment and sludge.

- 1) Report PDMS present in extraction blank in μg .

- 2) Report PDMS concentration in environmental samples in $\mu\text{g PDMS/g dry weight of sediment}$. Wet weight concentration may also be reported, but wet:dry ratios should then be given.

APPENDIX 2: STANDARD PROCEDURE FOR A SHORT-TERM GROWTH AND SURVIVAL ASSAY WITH THE MIDGE *CHIRONOMUS TENTANS*

Aquatic Toxicology Laboratory Standard Operating Procedure

Department of Zoology

Michigan State University

Title: Short-term growth and survival assay with the midge *C. tentans*.

K.S. Henry

Description of Method:

The recommended 10-d sediment toxicity test with *C. tentans* must be conducted at 23 degrees C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux. Test chambers are 300 mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten third instar midges are used to start a test. The numbers of replicates/treatment depends on the objective of the test. Eight replicates are recommended for routing testing. Midges in each test chamber are fed 1.0 mL of a 4 g/L suspension of Tetramin daily. Each chamber receives 2 volume additions/d of overlying water. Water renewals may be manual or automated, though we usually use manual methods in our lab. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. We normally use 30% well water/70% reverse osmosis purified or 70% ultrapure for our cultures and tests. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected.

Referenced Documents:

- Ankley, G.T., D.A. Benoit, R.A. Hoke, E.N. Leonard, C.W. West, G.L. Phipps, V.R. Mattson, and L.A. Anderson. 1993. Development and evaluation of test methods for benthic invertebrates and sediments: Effects of flow rate and feeding on water quality and exposure conditions. *Arch. Environ. Contam. Toxicol.* 25:12-19.
- Ingersoll, C.G. and M.K. Nelson. 1990. Testing sediment toxicity with *Hyaella azteca* (Amphipoda) and *Chironomus riparius* (Diptera). In: W.G. Landis and W.H. van der Schalie (eds.), *Aquatic toxicology and risk assessment*, 13th volume. ASTM STP 1096. Philadelphia, PA.
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- Reynoldson, T.B., K.E. Day, C. Clarke, D. Milani. 1994. Effect of indigenous animals on chronic endpoints in freshwater sediment toxicity tests. *Environ. Contam. Toxicol.* 13:973-977.
- Sadler, W.O. 1935. Biology of the midge *Chironomus tentans* Fabricus, and methods for its propagation. *Mem. Cornell Univ. Agric. Exp. Stat.* 173. 25 pp.
- USEPA. 1994. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA-600/R-94/024. Environmental Research Laboratory, Duluth, Minnesota. 133 p.

Procedure:

Preparation of Test Chambers

The day before the sediment test is started (Day -1) each sediment should be thoroughly mixed and added to the test chambers. Sediment should be visually inspected to judge the degree of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components, and sediment should be thoroughly mixed. If a quantitative measure of homogeneity is required, replicate sub-samples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size. This can be done in the soil analysis lab in the Crop and Soil Science Department on MSU's campus.

Preparation of Test Chambers

Overlying water is added to the chamber in a manner that minimizes suspension of sediment. This is best accomplished by slowly pouring the water onto a Styrofoam disk placed on top of the sediment to dissipate the force of the water. Renewal of overlying water is started on day -1 by performing a water exchange after all replicates for the test have been set up. A test begins when the organisms are added to the test chambers (day 0).

Renewal of Overlying Water

Two volume additions of overlying water should be delivered to each test chamber daily over the course of the test. Routine water quality parameters will be measured at regular intervals during the test (see below). The number of replacements per day should be increased if hardness, alkalinity or ammonia concentrations in the overlying water vary by more than 50% among treatments, or if oxygen drops to less than 30% saturation. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediments will generally still receive a substantial

proportion of a contaminant dose directly from the whole sediment and/or from the interstitial water. The water delivery system should be calibrated and run for a few days before a test is started to verify that the system is functioning properly.

Acclimation of Test Organisms and Placement into Test Chambers

Test organisms should be cultured and tested at 23°C in the same water type. However, if a sufficient supply of culture water is not available for the exposure, acclimation of test organisms to the test water is not required. Performance standards for this test are calibrated to the response of midges at 23°C. If the test must be run at a different temperature, then the organisms should be acclimated at a rate of 1°C every 2 h to prevent thermal shock.

Test organisms should be disturbed as little as possible. Midges should be introduced into the overlying water below the air-water interface. Test organisms can be pipetted directly into overlying water (Ankley et al. 1993). In our lab we typically place 5 larvae into culture water in 30 mL plastic cups, randomize these cups, and float them in the exposure chambers for 15 min before dumping the contents into the chambers.

Feeding

Each beaker receives a daily addition of 1.0 mL of 4g/L Tetramin. Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 40% of saturation during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in

all treatments. Detailed records of feeding rates and appearance of the sediment should be maintained daily.

Overlying Water Quality

All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Hardness, alkalinity, pH, conductivity, and ammonia in the overlying water within a treatment should not vary by more than 50% during a test.

Dissolved oxygen should be measured daily and should be between 40 and 100% saturation. If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Aeration can be used to maintain dissolved oxygen in the overlying water above 40% saturation, though increasing the number of water exchanges per day is preferable. Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath should also be continuously monitored using a max/min thermometer. Usually running the water bath in the fiberglass tanks at 25°C will result in an appropriate exposure chamber temperature (i.e. 23°C). The daily mean test temperature must be $23 \pm 1^\circ\text{C}$. The instantaneous temperature must always be $23 \pm 3^\circ\text{C}$.

Ending a Test

Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. A #25 (710µm mesh) sieve can be used to isolate midges from sediment. Surviving test organisms can be counted and placed into weigh pans or preserved in 8% sugar formalin solution for growth measurements. The sugar-formalin solution is made by adding 120 g of sugar to 80 mL of formalin, and bringing the solution up to 1 L with reverse osmosis water. This solution is mixed 1:1 with sample water to preserve organisms.

Test Data

Ash-free dry weight (AFDW) and survival are the endpoints measured at the end of the 10 d test. The duration of the test starting with third instar is not long enough to determine emergence of adults. Traditionally, dry weight (DW) has been used for the larval growth endpoint on midge tests. However, it has been shown that the grain size of sediments influences the amount of sediment that *C. tentans* larvae ingest and retain in their gut. As a result, in finer-grain sediments, a substantial portion of the measured dry weight may be comprised of sediment rather than tissue. While this may not represent a strong bias in tests with identical grain size distributions in all treatments, most field

assessments are likely to have varying grain size among sights. This will likely create differences in dry weight among treatments that are not reflective of true somatic growth. For this reason, it has been suggested that weight of midges should be measured as ash-free dry weight (AFDW) instead of dry weight. AFDW will more directly reflect actual differences in tissue weight by reducing the influence of sediment in the gut.

To find AFDW, all living larvae per replicate are combined in a pre-ashed aluminum weigh boat and dried at 60°C for 24h. The samples should be cooled in a dessicator and weighed to the nearest 0.01 mg to obtain mean weights per surviving organism per replicate. When weighing, remove only 4 weigh boats from the dessicator at a time and weigh them. When done, remove 4 more and so on until all are weighed. The dry pans will absorb water out of the air and increase in weight, so they should be weighed as soon as possible after removal from the dessicator. The dried larvae in the pan are then ashed at 550°C for 2h. The pan with the ashed larvae is then re-weighed and the tissue mass of the larvae is determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan. In rare instances, where preservation is required, an 8% sugar-formalin solution can be used to preserve samples. The sugar-formalin solution is made by adding 120 g of sugar to 80 mL of formalin, and bringing the solution up to 1 L with DI water. This solution is mixed 1:1 with sample water to preserve organisms. Sugar formalin is preferable to alcohol for preservation because it does not cause as much shrinkage as EtOH. Average AFDW of *C. tentans* in control sediments must be at least 0.48 mg at the end of the test. Head capsule width can also be measured on surviving midges at the end of a test before AFDW is determined, though we do not do this routinely.

Influence of Indigenous Organisms

The influence of indigenous organisms on the response of *C. tentans* has not been well studied, though survival of *C. riparius* was not reduced by the presence of oligochaetes in sediments (Reynoldson et al. 1994). Therefore, it is important to determine the number and biomass of indigenous organisms in field-collected sediment in order to better interpret growth data (Reynoldson et al. 1994). Furthermore, presence of predators may also influence the response of test organisms in sediment (Ingersoll and Nelson 1990).

Table 1. Test conditions for conducting a 10 d sediment toxicity test with *Chironomus tentans*.

Parameter	Conditions
1. Test Type	Whole-sediment toxicity test with renewal of overlying water
2. Temperature	23±1°C
3. Illuminance	About 500 to 1000 lux
4. Photoperiod	16L:8D
5. Test chamber	300 mL high-form lipless beakers
6. Sediment volume	100 mL
7. Overlying water volume	175 mL
8. Age of organisms	Third instar larvae
9. Number of organisms/chamber	10
10. Number of replicate chambers/treatment	8 for routine testing
11. Feeding	Tetramin goldfish food, fed 1.0 mL daily to each test chamber
12. Aeration	None unless DO in overlying water drops below 2.5 mg/L
13. Overlying water	Preferably culture water. Well water, surface water, or site water may also be used.
14. Test chamber cleaning	Occasionally the outside of the screen may be brushed gently with a toothbrush.
15. Overlying water quality	Hardness, alkalinity, conductivity, pH and ammonia at the beginning and end of a test (day 0 and day 10). Temperature and DO daily.
16. Test duration	10 d
17. Endpoints	Survival and growth
18. Test acceptability	Minimum mean control survival of 70% and mean weight per surviving control organism of 0.6 mg on day 10 and suggested performance-based criteria (see below).

Table 2. General activity schedule for conducting a 10 d sediment toxicity test with *Chironomus tentans*.

Day	Activity
-14	Isolate adults for production of egg masses
-13	Place newly deposited egg masses into hatching dishes (crystallizing dishes with water)
-12	A larval rearing tank is prepared by filling a 5 gal fish tank 1/3 full and adding 2 cups of ground paper towel
-11	Examine egg masses for hatching success. If egg masses have hatched, transfer larvae and eggs into the larval rearing tank. Feed midges with 20g/L Tetramin suspension. It is best to start several tanks if enough egg masses are available.
-9 to -1	Feed and observe midges.
-1	Add sediment into each test chamber, place chambers into exposure system, feed each chamber, and start renewing overlying water.
0	Measure total water quality (pH, temperature, DO, hardness, alkalinity, conductivity, ammonia). Isolate 3 rd instar larvae from the culture tanks. Add 1.0 mL of Tetramin (4.0 g/L) into each test chamber. Transfer 10 larvae into each test chamber. Release organisms under the surface of the water.
1 to 9	Add 1.0 mL of Tetramin (4.0 g/L) to each test beaker. Measure temperature and DO daily. Observe and record behavior of test organisms.
10	Measure temperature, DO, pH, hardness, alkalinity, conductivity and ammonia. End the test by collecting the amphipods with a #25 mesh sieve (710 μ m mesh). Measure weight of surviving larvae.

Table 3. Test acceptability requirements for conducting a 10 d sediment toxicity test with *Chironomus tentans*.

The following performance criteria for the short-term test with <i>C. tentans</i> have been recommended by the EPA:	
1.	Tests must be started with 3 rd instar and younger larvae.
2.	Average survival of <i>C. tentans</i> in the control sediment must be $\geq 70\%$ at the end of the test.
3.	Average size of <i>C. tentans</i> in the control sediment must be at least 0.6 mg at the end of the test.
4.	Hardness, alkalinity, pH, and ammonia in the overlying water typically should not vary by more than 50% during the sediment exposure.
B. Additional Requirements	
1.	All organisms in a test must be from the same source.
2.	It is desirable to start tests as soon as possible after collection of sediment from the field.
3.	Negative-control sediment must be included in a test.
4.	Test organisms should be cultured and tested at 23°C.
5.	The mean of the daily test temperature must be 23±1°C. The instantaneous temperature must be 23±3°C.

APPENDIX 3: STANDARD PROCEDURE FOR A SHORT-TERM GROWTH AND SURVIVAL ASSAY WITH THE AMPHIPOD *HYALELLA AZTECA*

Aquatic Toxicology Laboratory Standard Operating Procedure

Department of Zoology

Michigan State University

Title: Short-term growth and survival assay with the amphipod *Hyallela azteca*.

K.S. Henry

Description of Method:

The recommended 10-d sediment toxicity test with *H. azteca* must be conducted at 23 degrees C with a 16L:8D photoperiod at an illuminance of about 500 to 1000 lux. Test chambers are 300 mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten 7-to 14-d old amphipods are used to start a test. The numbers of replicates/treatment depends on the objective of the test. Eight replicates are recommended for routine testing. Amphipods in each test chamber are fed 1.5 mL of YCT food daily. Each chamber receives 2 volume additions/d of overlying water. Water renewals may be manual or automated, though we usually use manual methods in our lab. Overlying water can be culture water, well water, surface water, site water, or reconstituted water. We normally use 30% well water for our cultures and tests. For site-specific evaluations, the characteristics of the overlying water should be as similar as possible to the site where sediment is collected.

Referenced Documents:

- Environment Canada. 1997. Biological test method: Test for survival and growth in sediment using the freshwater amphipod *Hyaella azteca*. EPS 1/RM/33. Method Development and Application Section. 123 p.
- Ingersoll, C.G. and M.K. Nelson. 1990. Testing sediment toxicity with *Hyaella azteca* (Amphipoda) and *Chironomus riparius* (Diptera). In: W.G. Landis and W.H. van der Schalie (eds.), Aquatic toxicology and risk assessment, 13th volume. ASTM STP 1096. Philadelphia, PA. pp 93-109.
- Kemble, N.E., W.G. Brumbaugh, E.L. Brunson, F.J. Dwyer, C.G. Ingersoll, D.P. Monda, and D.F. Woodward. 1994. Toxicity of metal-contaminated sediments from the Upper Clark Fork River, MT, to aquatic invertebrates in laboratory exposures. Environ. Toxicol. Chem. 13:1985-1997.
- Reynoldson, T.B., K.E. Day, C. Clarke, D. Milani. 1994. Effect of indigenous animals on chronic endpoints in freshwater sediment toxicity tests. Environ. Contam. Toxicol. 13:973-977.
- USEPA. 1994. Method for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA-600/R-94/024. Environmental Research Laboratory, Duluth, Minnesota. 133 p.

Procedure:

Preparation of Test Chambers

The day before the sediment test is started (Day -1) each sediment should be thoroughly mixed and added to the test chambers. Sediment should be visually inspected to judge the degree of homogeneity. Excess water on the surface of the sediment can indicate separation of solid and liquid components. If a quantitative measure of

homogeneity is required, replicate sub-samples should be taken from the sediment batch and analyzed for TOC, chemical concentrations, and particle size. This can be done in the soil analysis lab in the Crop and Soil Science Department on MSU's campus.

Preparation of Test Chambers

Overlying water is added to the chamber in a manner that minimizes suspension of sediment. This is best accomplished by slowly pouring the water onto a Styrofoam disk placed on top of the sediment to dissipate the force of the water. Renewal of overlying water is started on day -1 by performing a water exchange after all replicates for the test have been set up. A test begins when the organisms are added to the test chambers (day 0).

Renewal of Overlying Water

Two volume additions of overlying water should be delivered to each test chamber daily over the course of the test. Routine water quality parameters will be measured at regular intervals during the test (see below). The number of replacements per day should be increased if hardness, alkalinity or ammonia concentrations in the overlying water vary by more than 50% among treatments, or if oxygen drops to less than 30% saturation. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediments will generally still receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water. The water delivery system should be calibrated and run before a test is started to verify that the system is functioning properly.

Acclimation of Test Organisms and Placement into Test Chambers

Test organisms should be cultured and tested at 23°C in the same test water. However, if a sufficient supply of culture water is not available for the exposure, acclimation of test organisms to the test water is not required. Performance standards for this test are calibrated to the response of amphipods at 23°C. If the test must be run at a different temperature, then the organisms should be acclimated at a rate of 1°C every 2 h to prevent thermal shock.

Test organisms should be disturbed as little as possible. To start a test, 7 to 14 d old amphipods are collected with a Pasteur pipette from the bottom of a crystallizing dish with the aid of a dissecting microscope. Test organisms are pipetted very gently directly into the overlying water of the exposure chambers. Care should be taken to release them under the surface of the water. Alternatively, we often place 5 amphipods into culture water in 30 mL plastic cups, randomize these cups, and float them in the exposure chambers for 15 min before dumping the contents into the chambers.

Feeding

Each beaker receives a daily addition of 1.5 mL of yeast, cerophyll, trout chow suspension (i.e. YCT). Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 40% of saturation during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in all treatments. Detailed records of feeding rates and appearance of the sediment should be maintained daily.

Overlying Water Quality

All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are usually not visible during the exposure. The operation of the exposure system should be monitored daily.

Conductivity, hardness, pH, alkalinity, and ammonia should be measured in all treatments at the beginning and end of a test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water. Hardness, alkalinity, pH, conductivity, and ammonia in the overlying water with a treatment should not vary by more than 50% during a test.

Dissolved oxygen should be measured daily and should be between 40 and 100% saturation. If a probe is used to measure dissolved oxygen in overlying water, it should be thoroughly inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination. Aeration can be used to maintain dissolved oxygen in the overlying water above 40% saturation, though increasing the number of water exchanges per day is preferable. Dissolved oxygen and pH can be measured directly in the overlying water with a probe.

Temperature should be measured at least daily in at least one test chamber from each treatment. The temperature of the water bath should also be continuously monitored using a max/min thermometer. Usually running the water bath in the fiberglass tanks at

25°C will result in an appropriate exposure chamber temperature (i.e. 23°C). The daily mean test temperature must be $23 \pm 1^\circ\text{C}$. The instantaneous temperature must always be $23 \pm 3^\circ\text{C}$.

Ending a Test

Any of the surviving amphipods in the water column or on the surface of the sediment can be pipetted from the beaker before sieving the sediment. Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. A #25 sieve (710 μm mesh) can be used to isolate amphipods from sediment. Alternatively, Kemble et al. (1994) recommended sieving sediment using the following procedure: (1) pour about half of the overlying water through a #50 (300 μm) U.S. Standard mesh sieve, (2) swirl the remaining water to suspend the upper 1 cm of sediment, (3) pour this slurry through the #50 mesh sieve and wash the contents of the sieve into an examination pan. Surviving test organisms should be counted and preserved in 8% sugar formalin solution for growth measurements. The sugar-formalin solution is made by adding 120 g of sugar to 80 mL of formalin, and bringing the solution up to 1 L with RO water. This solution is mixed 1:1 with sample water to preserve organisms.

Test Data

Survival is the primary endpoint recorded at the end of the 10-d sediment toxicity test with *H. azteca*. Measuring growth is optional; however, growth of amphipods may be a more sensitive toxicity endpoint than survival (e.g. Kemble et al. 1994). The duration of the 10-d test started with 7- to 14- old amphipods is not long enough to determine sexual maturation or reproductive effects, though the multi life stage test does consider these endpoints.

Amphipod body length (± 0.1 mm) can be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface. Antennal segment number can also be used to estimate length or weight of amphipods. Wet or dry weight measurements have also been used to estimate growth of *H. azteca*, and in our lab we normally use dry weight. Dry weight of amphipods should be determined by pooling all living organisms from a replicate into a homemade aluminum "weigh boat" about 1 cm in diameter. Samples are covered and dried at 60°C for 24 h. The samples should be cooled in a dessicator and weighed to the nearest 0.01 mg to obtain mean weights per surviving organism per replicate. When weighing, remove only 4 weigh boats from the dessicator at a time and weigh them. When done, remove 4 more and so on until all are weighed. The dry pans will absorb water out of the air and increase in weight, so they should be weighed as soon as possible after removal from the dessicator.

Influence of Indigenous Organisms

Survival of *H. azteca* in 28-d tests was not reduced in the presence of oligochaetes in sediment samples (Reynoldson et al. 1994). However, growth of amphipods was reduced when high numbers of oligochaetes were placed in a sample. Therefore, it is important to determine the number and biomass of indigenous organisms in field-collected sediments in order to better interpret growth data (Reynoldson et al. 1994). Furthermore, presence of predators may also influence the response of test organisms in sediment (Ingersoll and Nelson 1990).

Table 1. Test conditions for conducting a 10 d sediment toxicity test with *Hyalella azteca*.

Parameter	Conditions
1. Test Type	Whole-sediment toxicity test with renewal of overlying water
2. Temperature	23±1°C
3. Illuminance	About 500 to 1000 lux
4. Photoperiod	16L:8D
5. Test chamber	300 mL high-form lipless beakers
6. Sediment volume	100 mL
7. Overlying water volume	175 mL
8. Age of organisms	7 to 14 d old at the start of the test
9. Number of organisms/chamber	10
10. Number of replicate chambers/treatment	8 for routine testing
11. Feeding	YCT food, fed 1.5 mL (1800 mg/L stock) daily to each test chamber
12. Aeration	None unless DO in overlying water drops below 2.5 mg/L
13. Overlying water	Preferably culture water. Well water, surface water, or site water may also be used.
14. Test chamber cleaning	Occasionally the outside of the screen may be brushed gently with a toothbrush.
15. Overlying water quality	Hardness, alkalinity, conductivity, pH and ammonia at the beginning and end of a test (day 0 and day 10). Temperature and DO daily.
16. Test duration	10 d
17. Endpoints	Survival. Growth optional.
18. Test acceptability	Minimum mean control survival of 80% on day 10 and suggested performance-based criteria.

Table 2. General activity schedule for conducting a 10 d sediment toxicity test with *Hyalella azteca*.

Day	Activity
-7	Separate known-age amphipods from the cultures and place in breeding chambers.
-6 to -1	Feed and observe isolated amphipods.
-1	Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water.
0	Measure total water quality (pH, temperature, DO, hardness, alkalinity, conductivity, ammonia). Transfer ten 7 to 14 d old amphipods into each test chamber. Release organisms under the surface of the water. Add 1.5 mL of YCT into each test chamber.
1 to 9	Add 1.5 mL of YCT to each test beaker. Measure temperature and DO daily. Observe and record behavior of test organisms.
10	Measure temperature, DO, pH, hardness, alkalinity, conductivity and ammonia. End the test by collecting the amphipods with a #40 mesh sieve (425 μ m mesh).

Table 3. Test acceptability requirements for conducting a 10 d sediment toxicity test with *Hyalella azteca*.

A. The following performance criteria for the short-term test with <i>H. azteca</i> have been recommended by the EPA:	
1.	Age of <i>H. azteca</i> at the start of the test should be 7 to 14 d old.
2.	Average survival of <i>H. azteca</i> in the control sediment on day 28 should be greater than or equal to 80%.
3.	Hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the sediment exposure.
B. Additional Requirements	
1.	All organisms in a test must be from the same source.
2.	It is desirable to start tests as soon as possible after collection of sediment from the field.
3.	Negative-control sediment must be included in a test.
4.	Test organisms should be cultured and tested at 23°C.
5.	The mean of the daily test temperature must be 23±1°C. The instantaneous temperature must be 23±3°C.

APPENDIX 4: STANDARD PROCEDURE FOR A WHOLE LIFE CYCLE ASSAY WITH THE MIDGE *CHIRONOMUS TENTANS*

Aquatic Toxicology Laboratory Standard Operating Procedure

Department of Zoology

Michigan State University

Title: Whole life cycle assay with the midge *Chironomus tentans*.

K.S. Henry

Description of Method:

First instar larvae of the midge *Chironomus tentans* are placed into exposure chambers shortly after leaving their egg masses. The test continues through emergence, reproduction, and hatching of the F₁ generation. Survival is determined at 20 d and at the end of the test. Growth is determined at 20 d, which corresponds to the growth endpoint in the short-term test started with 10 d old larvae. From day 23 to the end of the test, emergence and reproduction are monitored daily. The number of eggs per female is determined for each egg case, which is incubated to determine hatching success. Each treatment is ended when no emergence occurs for 7 d in that treatment. The total length of the test is normally 50 to 60 d.

Referenced Documents:

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Procedure:

Test Conditions

Conditions for conducting a whole life stage sediment toxicity test with *C. tentans* are summarized in Table 1. An activity schedule is give as Table 2. This test is conducted at 23°C with a 16L:8D photoperiod. Test chambers are 300 mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water (Benoit beakers). Each test chamber receives 2 volume additions/d of overlying water. Water renewals are

performed manually or using an automatic dispenser system. Overlying water should be from a source that has been shown to support reproduction of *C. tentans* in culture. Reconstituted water should be avoided when possible.

For routine testing, a total of 16 replicates, each containing 12 larvae are used for each treatment. Assignment of beakers for the 16 replicates is as follows: On test day -1 12 replicates are set up of which 4 will be used for the 20 d growth and survival endpoints and 8 for determination of emergence and reproduction. Male *C. tentans* typically begin emerging 4 to 7 d before females. To permit overlap of breeding individuals, an additional 4 beakers are set up on test day 10 for production of auxiliary males, which are thus available during the prime female emergence period for each sediment treatment. Midges in each test chamber are fed 1.5 mL of a 4 g/L Tetramin suspension daily. Endpoints monitored include 20 d survival and growth, emergence, reproduction, and egg viability.

Specialized Equipment

Specialized equipment required for this test includes emergence traps, reproduction/oviposit (R/O) chambers, an aspirator, and an adult collector dish. Emergence traps are constructed from 120 mL plastic cups with the bottom removed and replaced with a disc of fiberglass window screen. The traps fit over the rim of the 300 mL beakers and trap the adult midges as they emerge. The emergence traps are also used as R/O chambers. To serve in this application, the chambers are placed in a 100 x 20 mm petri dish containing approximately 50 mL of culture water. At the time of the first emergence in each replicate, a corresponding R/O unit is initiated and placed on the shelf within the test system for monitoring (a single R/O chamber is used to hold all adults that emerge from a replicate).

An aspirator is constructed to aid in the collection and transfer of adults. The aspirator consists of a 60 cc syringe with detachable aspirator unit. The barrel end of the syringe is cut off and a retainer, used to hold the emergence traps and R/O units, is attached. The retainer is constructed from a 7 cm diameter plastic lid, obtained from a 270 mL widemouth glass jar and a no. 10.5 rubber stopper. A hole is bored through the center of both the lid and rubber stopper and the lid is glued onto the stopper with silicone caulking. The open end of the syringe is then pushed through the hole of the retainer/rubber stopper unit such that the open edge of the syringe is aligned with the bottom of the inverted lid. A detachable tubing unit can then be fixed on the open end of the syringe for collection of midge adults. This tubing unit is made of a no. 5.5 rubber stopper into which two 7 mm diameter holes are bored. A fire-polished glass collector tube (6 mm ID) 16 cm in length is placed into one of the two holes while a 4 cm length of the same glass tubing is placed into the other hole. Both tubes should be flush with the bottom of the rubber stopper after insertion. A disk cut of fiberglass window screen is fit over the lower end of the short glass tube to prevent adults from being sucked into the inhalant siphon of the aspirator. A 70 cm length of tygon tubing (6 mm ID, 9.5 mm OD) is attached onto the opposite end of the short glass piece.

An adult collector dish, used in conjunction with the collector/transfer apparatus, is constructed from a 100 x 20 mm petri dish. At the center of this dish, a 2.54 cm access hole is drilled and covered with fiberglass window screen attached with silicone caulking. Two slits within the screen, cut at 90° angles in the form of an "x" provide access (using the collector tube) to the emergence traps or R/O units.

Collection of Egg Masses and Hatching of Eggs

Cultures should be maintained so that a minimum of 12 egg masses are produced on day -3. This will require the collection of approximately 25 female and 10 male adult midges on day -4. On day -3, 8 of the most uniform egg masses should be separated from the total collected, transferred to a petri dish with culture water, and incubated at 23°C. Hatching typically begins around 48 h post-oviposition and the majority of larvae leave the egg masses 24 h after the first hatch.

Hatching of eggs should be complete by about 72 h. After the first 24 h period with larvae hatched, transfer the egg masses from the incubation dish to another dish with clean test water. Larvae that have already left the egg mass in the first incubation dish are discarded in order to reduce the age range of individuals used in the test. The action of transferring the egg case stimulates the remaining larvae to leave the egg mass within a few hours. These larvae are used to start the test.

Preparation of Test Chambers

The day before the test is started (day -1) each sediment should be thoroughly homogenized and added to the test chambers. Sediment homogenization is best performed by mixing the sediments in a bucket with a drill-mounted stainless steel paddle. A stainless steel spoon should then be used to transfer 100 mL of sediment into each beaker. Any sediment which is accidentally smeared on the sides of the beaker above the 100 mL mark should be removed with a paper towel. Otherwise this sediment may serve as refuge for the invertebrates if they try to avoid the main mass of sediment in the bottom of the beaker.

Overlying water is added to the chamber in a manner that minimizes suspension of sediment. This is best accomplished by slowly pouring the water onto a Styrofoam disk

placed on top of the sediment to dissipate the force of the water. Renewal of overlying water is started on day -1 by performing a water exchange after all replicates for the test have been set up. A test begins when the organisms are added to the test chambers (day 0).

Renewal of Overlying Water

Two volume additions of overlying water should be delivered to each test chamber daily over the course of the test. Routine water quality parameters will be measured at regular intervals during the test (see below). The number of replacements per day should be increased if hardness, alkalinity or ammonia concentrations in the overlying water vary by more than 50% among treatments, or if oxygen drops to less than 30% saturation. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediments will generally still receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water. The water delivery system should be calibrated and run before a test is started to verify that the system is functioning properly.

Acclimation of Test Organisms and Placement into Test Chambers

Test organisms must be cultured and tested at 23°C in the same test water. However, if a sufficient supply of culture water is not available for the exposure, acclimation of test organisms to the test water is not required. Performance standards for this test are calibrated to the response of midges at 23°C. If the test must be run at a different temperature, then the organisms should be acclimated at a rate of 1°C every 2 h to prevent thermal shock. In addition, testing at temperatures other than 23°C need to be

preceded by studies to determine expected performance under other than standard conditions.

Test organisms should be disturbed as little as possible. To start a test, larvae are collected with a Pasteur pipette from the bottom of the incubation dish with the aid of a dissecting microscope. Test organisms are pipetted very gently directly into the overlying water of the exposure chambers. Care should be taken to release them under the surface of the water. If possible, all larvae needed for the test should be placed into beakers within 4 h of transfer of egg masses from the first to the second incubation dish. If all emerged larvae have been transferred from the incubation dish to the exposure chambers, it may be necessary to wait for additional larvae to leave their egg masses. To minimize this possibility, the test should be initiated with a sufficient number of egg masses of similar age.

Feeding

Each beaker receives a daily addition of 1.5 mL of Tetramin (4 g/L, note it has recently been suggested that this volume be reduced to 1.0 mL/day to reduce the chance of DO sags; USEPA 1999). Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 2.5 ppm during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in all treatments.

Overlying Water Quality

All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

Conductivity, hardness, alkalinity, and ammonia should be measured in all treatments at the beginning of the test and at least weekly until the end of the test. DO and pH should be taken at the beginning of a test and at least three times a week until the end of the test. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water.

Routine chemistries on day 0 should be taken before organisms are placed in the test beakers. DO and pH can be measured directly in the overlying water in an effort to accurately measure concentrations of DO. The DO probe should be inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination.

It is likely that DO concentrations will drop significantly during the test. Based on tests run at the EPA and elsewhere, concentrations above 1.5 ppm do not seem to have an effect on midge survival and development. However, if the DO drops below 2.5 ppm for more than one day, then water replacements should be increased or aeration of all exposure chambers should begin. Occasional brushing of the outsides of the screens on the exposure chambers will help maintain the exchange of water during renewals.

Temperature should be checked at least daily in at least one exposure chamber from each treatment. The temperature of the water bath or the exposure chamber should be continuously monitored with a max/min thermometer. The daily mean test temperature must be $23 \pm 1^{\circ}\text{C}$. The instantaneous temperature must always be $23 \pm 3^{\circ}\text{C}$.

Monitoring Survival and Growth

At 20 d, 4 of the initial 12 replicates are randomly selected for use in growth and survival measurements. A #40 sieve (425 μm mesh) should be used to isolate larvae from the sediment. Any immobile organisms isolated from the sediment surface or from sieved material should be considered dead. Often midge larvae tend to lose their coloration within 15 to 20 min of death and may become rigidly elongate. Surviving larvae are kept separated by replicate for weight measurements; if pupae are recovered, these are included in survival data but not included in the growth data.

Traditionally, dry weight (DW) has been used for the larval growth endpoint on midge tests. However, it has been shown that the grain size of sediments influences the amount of sediment that *C. tentans* larvae ingest and retain in their gut. As a result, in finer-grain sediments, a substantial portion of the measured dry weight may be comprised of sediment rather than tissue. While this may not represent a strong bias in tests with identical grain size distributions in all treatments, most field assessments are likely to have varying grain size among sights. This will likely create differences in dry weight among treatments that are not reflective of true somatic growth. For this reason, it has been suggested that weight of midges should be measured as ash-free dry weight (AFDW) instead of dry weight. AFDW will more directly reflect actual differences in tissue weight by reducing the influence of sediment in the gut.

To find AFDW, all living larvae per replicate are combined in a pre-ashed aluminum weigh boat and dried at 60°C for 24h. The samples should be cooled in a dessicator and weighed to the nearest 0.01 mg to obtain mean weights per surviving organism per replicate. When weighing, remove only 4 weigh boats from the dessicator at a time and weigh them. When done, remove 4 more and so on until all are weighed. The dry pans will absorb water out of the air and increase in weight, so they should be weighed as soon as possible after removal from the dessicator. The dried larvae in the pan are then ashed at 550°C for 2h. The pan with the ashed larvae is then re-weighed and the tissue mass of the larvae is determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan. In rare instances, where preservation is required, an 8% sugar-formalin solution can be used to preserve samples. The sugar-formalin solution is made by adding 120 g of sugar to 80 mL of formalin, and bringing the solution up to 1 L with DI water. This solution is mixed 1:1 with sample water to preserve organisms. Sugar formalin is preferable to alcohol for preservation because it does not cause as much shrinkage as EtOH.

Monitoring Emergence

Emergence traps are placed on the reproductive replicates on day 20. Emergence traps for the auxiliary beakers are added at the corresponding 20 d time interval for those replicates. At 23°C, emergence in reference sediment typically begins on or about day 23 and continues for about 2 weeks. In contaminated sediments, the emergence period may be extended by several weeks.

Two categories are recorded for emergence: complete emergence and partial emergence. Complete emergence occurs when an organism has shed the pupal exuviae

completely and escapes the surface tension of the water. If complete emergence has occurred but the adult has not escaped the surface tension of the water, the adult will usually die within 24 h. Therefore, 24 h should elapse before this death is recorded. Partial emergence occurs when an adult has only partially shed the pupal exuviae. These adults will also die, which can be recorded after 24 h. Pupae at the sediment surface or the air-water interface may emerge successfully during the 24 h period. However, cannibalism of sediment-bound pupae by larvae may also occur. Data are recorded on the attached data sheets.

Collecting Adults for Reproduction

Adults are collected daily from individual traps using the aspirator and collector dish. With the collector dish nearby, the emergence trap is quickly moved from the beaker onto the dish. With the syringe plunger fully drawn, the glass collector tube is inserted through the screened access hole of the collector dish and the adults gently aspirated into the syringe barrel. Aspirated adults can easily be seen through the translucent plastic of the syringe. The detachable portion of the aspirator unit is then replaced with a reproduction/oviposit (R/O) chamber. This exchange can be facilitated by placing the thumb of the hand holding the syringe over the barrel entry port until the R/O chamber is in place. With the R/O chamber in place, and the plunger on the table top, the barrel of the syringe is pushed gently downward which forces the adults to move up into the R/O unit. Adults remaining on the transfer apparatus may be prodded into the R/O chamber by gently tapping the syringe. The transfer process is completed by quickly moving the R/O chamber to a petri dish containing overlying water. At all times during the transfer

process, it is important to ensure that the adults are stationary to minimize the possibility of escape.

At about day 33 until the end of the test, the auxiliary males may be needed to support reproduction in females. Males that emerge from the auxiliary male replicates are transferred to individual inverted R/O chambers. Each male may be used for mating with females from corresponding treatments for up to 5 d. Males may be used for breeding with more than one new emergent female. Males from a different replicate within the same sediment treatment may be paired with females of replicates where no males have emerged. Record data on data sheets.

Monitoring Reproduction

Each R/O unit is checked daily for dead adults and egg cases. Dead organisms are removed. In situations where many adults are contained within an R/O chamber, it may be necessary to assume that a dead adult is the oldest male or female in that replicate for the purpose of recording time to death. To remove dead adults and egg cases from the R/O chamber, one side of the chamber is carefully lifted just enough to permit the insertion of a transfer pipet or tweezers.

For each emerged female, at least one male, obtained from the corresponding reproductive replicate, from another replicate of that treatment, or from the auxiliary male beakers, is transferred into the R/O unit using an aspirator. Females generally remain sexually receptive up to 3 d if they have not already mated. Almost all females will oviposit within 1 d of fertilization; however, a few will require as long as 72 h to oviposit. A female will lay a single primary egg case, usually in the early morning. A second, generally smaller egg case may be laid; however these second egg cases are prone to

fungus and the viability of embryos is typically poor. These second egg cases do not need to be counted, or recorded, and the numbers of eggs are not included in the egg counts because eggs in the secondary egg cases typically have low viability.

Counting Eggs, Egg Case Incubation, and Hatch Determination

Primary egg cases from the R/O chamber are transferred to a separate and corresponding petri dish to monitor incubation and hatch. The number of eggs should be estimated in each egg case by using the ring count method: i) For each egg case, the mean number of eggs in five rings is determined; ii) these rings should be selected at about equal distances along the length of the egg case; iii) the number of eggs/ring multiplied by the number of rings in the egg case will provide an estimate of the total number of eggs. This can be done in about 5 min or less for each egg case. According to the EPA (USEPA 1999), accuracy of estimating versus a direct count method is very close (about 95%). The ring method is best suited to the symmetrically "C" shaped egg masses.

When the integrity of an egg case precludes estimation by the ring method (egg case is convoluted or distorted), the eggs must be counted directly. Each egg case is transferred to a petri dish with 2N sulfuric acid and left overnight. The acid dissolves the gelatinous matrix surrounding the eggs but does not affect the structural integrity of the eggs themselves. After digestion, the eggs can be counted by collecting them with a Pasteur pipet and spread across a microscope slide for counting under a dissecting microscope. Counting can be simplified by drawing a grid on the underside of the slide. Alternatively, they may be counted by removing them from the petri dish with a Pasteur pipet and counting the eggs with a push-button counter as they are collected into the

pipet. The direct count method requires a minimum of 10 min to complete and does not permit determination of hatching success.

Following estimated egg counts (ring count method), each egg case is transferred to a 60 x 15 mm plastic petri dish containing 15 mL overlying water and incubated at 23°C until hatching is complete. Although the time required to initiate hatching at this temperature is about 2 d, the period of time required to bring about complete hatch may be as long as 6 d. Therefore, hatching success is determined after 6 d of incubation. Hatching success is determined by subtracting the number of unhatched eggs remaining after the 6 d period from the number of eggs originally estimated for that egg case. Unhatched eggs either remain in the gelatinous egg case or are distributed on the bottom of the petri dish. Note that the unhatched eggs may decompose if the incubation temperature rises much above 23°C, so it is important that this temperature be maintained.

Test Termination

The quality of the sediments will determine the length of this test. In clean sediments, the test typically requires 40 to 50 d for completion. However, test duration will increase in the presence of environmental stressors, which act to reduce growth and delay emergence. Where a strong gradient of sediment contamination exists, emergence patterns between treatments will likely become asynchronous, in which case we have ended the test in our lab when there is no emergence from the control treatments for 7 d. However, it should be noted that the EPA has more recently suggested ended each treatment separately at a point which is determined by the performance of that treatment (USEPA 1999). If it is decided that each treatment will be ended separately, a treatment is terminated when no further emergence is recorded over a period of 7 d. In either case,

at test termination, all beakers of the treatment are sieved through a #40 mesh screen (425 μm) to recover remaining larvae, pupae, or pupal cases. If no emergence is recorded in a treatment at any time during the test, the treatment is ended once emergence in the control sediment has ended. In this case, you have probably chosen the wrong test for that sediment, and a short-term exposure with survival and growth might be more appropriate.

Interpretation of Results

Data Analyses

Endpoints measured in the *C. tentans* test include survival, growth, emergence, and reproduction. This section describes some general information from the EPA, the USFWS, etc. that will help in interpretation of results.

Age Sensitivity

Midges are perceived to be relatively insensitive organisms in toxicity assessments. This conclusion is based in great part on using the survival and growth of third and fourth instar animals in short-term exposures. The first and second instars of chironomids are more sensitive to contaminants than the third or fourth instars. For example, first instar *C. tentans* larvae were 6 to 27 times more sensitive than fourth instar larvae to acute copper exposure (Nebeker et al. 1984). Sediment tests must be started with uniform age and size midges because of the dramatic differences in sensitivity of midges by age.

Physical Characteristics of Sediment

Grain Size and Organic Matter

Larvae of *C. tentans* appear to be tolerant of a wide range of particle size conditions in substrates. Survival does not appear to be affected by grain size in natural sediments, sand substrates, or formulated sediments in both 10 d and long-term exposures.

Some studies have shown that growth of *C. tentans* is weakly correlated with sediment grain size composition. However, other studies by Paul Sibley at the University of Guelph found that the correlation between grain size and larval growth disappeared after accounting for inorganic material contained within larval guts, and he concluded that growth of midges was not related to grain size composition in either natural sediment or sand substrates. Avoiding confounding influences of gut contents on weight is the reason why AFDW is now recommended, as described above. Sibley also found that emergence, reproduction (in eggs/female), and hatch success were not affected by the particle size composition of substrates in chronic tests with *C. tentans*. The organic matter content of sediments does not appear to affect survival of *C. tentans* larvae, but the type and quantity of OM may be important for growth and should be considered if sediments tested are from different sources.

Influence of Indigenous Organisms

Survival of *C. riparius* was not reduced in the presence of a moderate number of oligochaetes in sediment samples, though growth was reduced when high numbers of oligochaetes were placed in the samples (Reynoldson et al. 1994). The presence of predators may also influence the response of test organisms in sediment (Ingersoll and Nelson, 1990).

Relative Endpoint Variability

According to the EPA, based on coefficient of variation (CV) determined from their standard control sediment (West Bearskin Lake), the following variability has been documented for the various endpoints in the *C. tentans* life cycle test: Survival (<20%),

growth as dry weight (<15%), emergence (<30%), reproduction as mean eggs/female (<20%), percent hatch (<10%).

Relative Endpoint Sensitivity

Measurement of sublethal endpoints (e.g. growth) can often provide a lot of information compared to acute endpoints (survival). Sibley et al. (1997) found that larval *C. tentans* exposed to a gradient of food stress did not experience significant effects on survival, yet did experience a significant reduction in growth and reproduction. Further, the proportion of larvae hatching in Sibley's study was high (>80%), and not systematically related to treatment, suggesting that percent hatch may be a relatively insensitive endpoint. We have come to much the same conclusions in our own lab studies.

Table 1. Test conditions for conducting a whole-life cycle assay with *Chironomus tentans*.

Parameter	Conditions
1. Test Type	Whole-sediment toxicity test with renewal of overlying water
2. Temperature	23±1°C
3. Illuminance	About 500 to 1000 lux
4. Photoperiod	16L:8D
5. Test chamber	300 mL high-form lipless beakers
6. Sediment volume	100 mL
7. Overlying water volume	175 mL
8. Age of organisms	< 24 h old larvae
9. Number of organisms/chamber	12
10. Number of replicate chambers/treatment	16 (12 at day -1 and 4 for auxiliary males on day 10).
11. Feeding	Tetramin food, fed 1.5 mL (4 g/L stock) daily to each test chamber starting on day -1
12. Aeration	None unless DO in overlying water drops below 2.5 mg/L
13. Overlying water	Preferably culture water. Well water, surface water, or site water may also be used.
14. Test chamber cleaning	Occasionally the outside of the screen may be brushed gently with a toothbrush.
15. Overlying water quality	Hardness, alkalinity, conductivity, and ammonia at the beginning and end of a test. Temperature daily. Dissolved oxygen (DO) and pH three times/week. Concentrations of DO should be measured more often if DO drops more than 1 mg/L since the previous measurement.
16. Test duration	About 40 to 50 d
17. Endpoints	20 day survival and growth; female and male emergence, adult mortality, number of egg masses deposited, number of eggs produced, and number of hatched eggs.
18. Test acceptability	Mean weights of organisms in controls at 20 d must be at least 0.6 mg/individual as dry wt. Or 0/48 mg/ind. as ash-free dry weight and should be at least 70% survival.

Table 2. General activity schedule for conducting a whole-life cycle assay with *Chironomus tentans*.

Day	Activity
-4	Start reproduction flask with cultured adults (1:3 male:female ratio).
-3	Collect egg masses and incubate at 23°C.
-1	Check egg cases for hatch and development. Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water. Add 1.5 mL Tetramin suspension to each chamber.
0	Measure total water quality (pH, temperature, DO, hardness, alkalinity, conductivity, ammonia). Transfer all egg cases to a crystallizing dish containing control water. Discard larvae that have already left the egg cases in the incubation dishes. Add 1.5 mL Tetramin suspension to each chamber before adding larvae. Add 12 larvae to each replicate beaker.
1- End	Add 1.5 mL Tetramin suspension to each beaker. Measure temperature daily. Measure pH and DO three times a week. If DO declines more than 1 mg/L between measurements, increase the number of water replacements or aerate all beakers. Measure hardness, alkalinity, conductivity, ammonia weekly.
6	For auxiliary male production, start reproduction flask with culture adults.
7-10	Set up schedule for auxiliary male beakers (4 replicates/treatment) same as that described above for day -3 through 0.
19	Ash weigh boats at 550°C for 2h in preparation for weight measurements. Store boats in a dessicator before use.
20	Randomly select 4 replicates from each treatment and sieve the sediment to recover larvae for growth and survival determination. Pool all living larvae per replicate and dry the sample at 60°C for 24 h. Install emergence traps on each of the remaining reproduction replicate beakers.
21	Samples with dried larvae are brought to room temperature in a dessicator and weighed to the nearest 0.01 mg. The pans are then ashed at 550°C for 2 h. Pans are then reweighed and the tissue mass of the larvae determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan.
23- End	On a daily basis, record emergence of males and females, pupal, and adult mortality, and time to death for previously collected adults. Each day, transfer adults from each replicate to a corresponding reproduction/ovoposition (R/O) chamber. Transfer each primary egg case from the R/O chamber to a corresponding petri dish to monitor incubation and hatch. Record each egg case oviposited number of eggs produced, and number of hatched eggs. If it is difficult to estimate the number of eggs in an egg case, used a direct count to determine the number of eggs; however the hatchability data will not be obtained for this egg case.
30	Place emergence traps on auxiliary male replicate beakers.
33- End	Transfer males emerging from the auxiliary male replicates to individual inverted petri dishes. The auxiliary males are used for mating with females from corresponding treatments from which most of the males are likely to have already emerged or in which no males emerged.
40- End	After 7 d of no recorded emergence in the control treatment, the test can be terminated by sieving the sediment to recover larvae, pupae, or pupal exuviae. Alternatively, each treatment can be ended independently when no emergence has occurred in that treatment for 7 d.

Table 3. Test acceptability requirements for a whole-life cycle assay with *Chironomus tentans*.

B. The following performance criteria for the whole life stage test with <i>C. tentans</i> have been recommended by the EPA:	
1.	Tests must be started with less than 1 d old larvae.
2.	Average survival of midges in the control sediment must be greater than or equal to 70% at the end of the test. Average dry weights should be ≥ 0.6 mg/individual, or ≥ 0.48 mg/individual AFDW.
3.	Based on round-robin and EPA lab results, larval survival is usually $> 76\%$, adult survival is $> 96\%$, and emergence is $> 60\%$. Time to death after emergence is < 6.5 d for males and < 5.1 d for females. The mean number of eggs per egg mass range from 906 to 1107 and the percent hatch ranges from 91 to 96%.
4.	Hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the test.
B. Additional Requirements	
1.	All organisms in a test must be from the same source.
2.	Negative-control sediment must be included in a test.
3.	Test organisms should be cultured and tested at 23°C .
4.	The mean of the daily test temperature must be $23 \pm 1^{\circ}\text{C}$. The instantaneous temperature must be $23 \pm 3^{\circ}\text{C}$.

APPENDIX 5: STANDARD PROCEDURE FOR A WHOLE LIFE CYCLE ASSAY WITH THE AMPHIPOD *HYALELLA AZTECA*

Aquatic Toxicology Laboratory Standard Operating Procedure

Department of Zoology

Michigan State University

Title: Whole life cycle assay with the amphipod *Hyaella azteca*.

Description of Method:

The sediment exposure portion of this assay starts with 7- to 8-d-old amphipods. On Day 28, amphipods are isolated from the sediment and placed in water-only chambers where reproduction is measured on Day 35 and 42. Typically, amphipods are first in amplexus at about Day 21 to 28 with release of the first brood between Day 28 to 42. Endpoints measured include survival (Day 28, 35 and 42), growth (as length or weight measured on Day 28 and 42), and reproduction (number of young/female produced from Day 28 to 42).

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Procedure:

Test Conditions

Conditions for evaluating sublethal endpoints in a sediment toxicity test with *H. azteca* are summarized in Table 1. A general activity schedule is outlined in Table 2. The 42-d sediment toxicity test with *H. azteca* is conducted at 23°C with a 16L:8D photoperiod at an illuminance of about 500 to 100 lux. Test chambers are 300-mL high-form lipless beakers containing 100 mL of sediment and 175 mL of overlying water. Ten amphipods in each test chamber are fed 1.0 mL of yeast, cerophyll, trout chow suspension (YCT) daily. Each test chamber receives 2 volume additions/d of overlying water. Water renewals are performed manually or using an automatic dispenser system. Overlying water should be from a source that has been shown to support reproduction of *H. azteca*

in culture. In our lab we usually use 30% well water and 70% reverse osmosis or ultrapure water. Reconstituted water should be avoided when possible.

The number of replicates and concentrations tested depends in part on the significance level selected and the type of statistical analysis. A total of 12 replicates, each containing ten 7- to 8-d-old amphipods, are usually tested for each sample. Starting the test with substantially younger or older organisms may compromise the reproductive endpoint. For the total of 12 replicates the assignment of beakers is as follows: 12 replicates are set up on Day -1 of which 4 replicates are used for 28-d growth and survival endpoints and 8 replicates for measurement of survival and reproduction on Day 35, and survival, reproduction, and growth on Day 42.

Preparation of Test Chambers

The day before the test is started (day -1) each sediment should be thoroughly homogenized and added to the test chambers. Sediment homogenization is best performed by mixing the sediments in a bucket with a drill-mounted stainless steel paddle. A stainless steel spoon should then be used to transfer 100 mL of sediment into each beaker. Any sediment which is accidentally smeared on the sides of the beaker above the 100 mL mark should be removed with a paper towel. Otherwise this sediment may serve as refuge for the invertebrates if they try to avoid the main mass of sediment in the bottom of the beaker.

Overlying water is added to the chamber in a manner that minimizes suspension of sediment. This is best accomplished by slowly pouring the water onto a Styrofoam disk placed on top of the sediment to dissipate the force of the water. Renewal of overlying water is started on day -1 by performing a water exchange after all replicates for the test

have been set up. A test begins when the organisms are added to the test chambers (day 0).

Renewal of Overlying Water

Two volume additions of overlying water should be delivered to each test chamber daily over the course of the test. Routine water quality parameters will be measured at regular intervals during the test. The number of replacements per day should be increased if hardness, alkalinity or ammonia concentrations in the overlying water vary by more than 50% among treatments, or if oxygen drops to less than 30% saturation. Although contaminant concentrations are reduced in the overlying water in water-renewal tests, organisms in direct contact with sediments will generally still receive a substantial proportion of a contaminant dose directly from either the whole sediment or from the interstitial water. The water delivery system should be calibrated and run before a test is started to verify that the system is functioning properly.

Acclimation of Test Organisms and Placement into Test Chambers

Test organisms must be cultured and tested at 23°C in the same test water. However, if a sufficient supply of culture water is not available for the exposure, acclimation of test organisms to the test water is not required. Performance standards for this test are calibrated to the response of amphipods at 23°C. If the test must be run at a different temperature, then the organisms should be acclimated at a rate of 1°C every 2 h to prevent thermal shock. In addition, testing at temperatures other than 23°C need to be preceded by studies to determine expected performance under other than standard conditions.

Test organisms should be disturbed as little as possible. To start a test, amphipods are collected with a Pasteur pipette from the bottom of a crystallizing dish with the aid of a dissecting microscope. Test organisms are pipetted directly into the overlying water of the exposure chambers. Care should be taken to release them under the surface of the water.

Feeding

Each beaker receives a daily addition of 1.0 mL of YCT. Suspensions of food should be thoroughly mixed before aliquots are taken. If excess food collects on the sediment, a fungal or bacterial growth may develop on the sediment surface, in which case feeding should be suspended for one or more days. A drop in dissolved oxygen below 2.5 mg/L during a test may indicate that the food added is not being consumed. Feeding should be suspended for the amount of time necessary to increase the dissolved oxygen concentration. If feeding is suspended in one treatment, it should be suspended in all treatments.

Overlying Water Quality

All chambers should be checked daily and observations made to assess test organism behavior such as sediment avoidance. However, monitoring effects on burrowing activity of test organisms may be difficult because the test organisms are often not visible during the exposure. The operation of the exposure system should be monitored daily.

Conductivity, hardness, alkalinity, and ammonia should be measured in all treatments at the beginning of the test and at least weekly until the end of the test. Total water quality should also be measured at the beginning and end of the reproductive phase

(day 29 and day 42). Conductivity should be measured weekly and DO and pH three times/week. Overlying water should be sampled just before water renewal from about 1 to 2 cm above the sediment surface using a pipet. It may be necessary to pool water samples from individual replicates. The pipet should be checked to make sure no organisms are removed during sampling of overlying water.

Routine chemistries on day 0 should be taken before organisms are placed in the test beakers. DO and pH can be measured directly in the overlying water in an effort to accurately measure concentrations of DO. The DO probe should be inspected between samples to make sure that organisms are not attached and should be rinsed between samples to minimize cross contamination.

It is likely that DO concentrations will drop significantly during the test, but should not run below 2.5 mg/L. If the DO drops below 2.5 ppm for more than one day, then water replacements should be increased or aeration of all exposure chambers should begin. Occasional brushing of the outsides of the screens on the exposure chambers will help maintain the exchange of water during renewals.

Temperature should be checked at least daily in at least one exposure chamber from each treatment. The temperature of the water bath or the exposure chamber should be continuously monitored with a max/min thermometer. The daily mean test temperature must be $23 \pm 1^{\circ}\text{C}$. The instantaneous temperature must always be $23 \pm 3^{\circ}\text{C}$.

Test Termination

Endpoints monitored include 28-d survival and growth of amphipods and 35-d and 42-d survival, growth, and reproduction (number of young/female) of amphipods. On day 28, 4 of the replicate beakers/sediment are sieved with a #40 mesh sieve (425-um mesh;

U.S. standard size sieve) to remove surviving amphipods. Animals in the water column or on the surface of the sediment can be pipetted from the beaker before sieving the sediment. The sediment in each beaker should be sieved in two separate aliquots (i.e. most of the amphipods will probably be found in the surface aliquot). Immobile organisms isolated from the sediment surface or from sieved material should be considered dead. Surviving amphipods from these 4 replicates are preserved in separate vials containing 8% sugar formalin solution if length of amphipods is to be measured. We do not always do length in our lab because of the fleeting availability of a digitizing microscope. The sugar formalin solution is prepared by adding 120 g of sucrose to 80 mL of formalin which is then brought to a volume of 1 L using deionized water. This stock solution is mixed with an equal volume of deionized water when used to preserve organisms. Sugar formalin is preferable to alcohol for preservation because it does not cause as much shrinkage as EtOH.

Growth of amphipods can be reported as either length or weight. Amphipod body length (± 0.1 mm) can be measured from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface. Dry weight of amphipods in each replicate can be determined on day 42 and for the archived samples on day 28. If both weight and length are to be determined, weight should be measured after length on the preserved samples. Dry weight of amphipods can be determined by: (1) transferring the archived amphipods from a replicate out of the sugar formalin solution into a crystallizing dish; (2) rinsing amphipods with deionized water; (3) transferring these rinsed amphipods to a pre-weighed aluminum micro-weigh boat (1 cm in diameter); (4) drying these samples for 24 h at 60 degrees C; and (5) weighing the pan and dried amphipods on a balance to the nearest

0.01 mg. Average dry weight of individual amphipods in each replicate is calculated from these data. Due to the small size of amphipods, caution should be taken during weighing (10 dried amphipods after a 28-d sediment exposure may weigh less than 2.5 to 3.5 mg). Weigh pans need to be carefully handled using powderless gloves and the balance should be calibrated with standard weights with each use. Use of small, homemade aluminum pans (e.g., 1 cm in diameter) will help reduce variability in measurements of dry weight. These may be constructed out of sheets of aluminum foil.

Historically, dry weight has been used for the growth endpoint for *C. tentans* and *H. azteca*. However, for *C. tentans*, this recommendation was changed to ash-free dry weight (AFDW) instead of dry weight, with the intent of reducing bias introduced by gut contents (Sibley et al. 1997). However, this recommendation was not extended to include *H. azteca* because the ash content of *H. azteca* is not greatly decreased by purging organisms in clean water before weighing, suggesting that sediment does not comprise a large portion of the overall dry weight. In addition, using AFDW further decreases an already small mass, potentially increasing measurement error.

On Day 28, the remaining 8 of the original 12 beakers/sediment are also sieved and the surviving amphipods in each sediment beaker are placed in 300 mL water-only beakers containing 150 to 275 mL of overlying water and a 5-cm x 5-cm piece of Nitex screen. Each water-only beaker receives 1.0 mL of YCT stock solution and about two volume additions of water daily.

Reproduction of amphipods is measured on Day 35 and Day 42 in the water-only beakers by removing and counting the adults and young in each beaker. On Day 35, the adults are then returned to the same water-only beakers. Adult amphipods surviving on

Day 42 are preserved in sugar formalin. The number of adult females is determined by simply counting the adult males (mature male amphipods will have an enlarged second gnathopod) and assuming all other adults are females. The number of females is used to determine number of young/female/beaker from Day 28 to Day 42. Growth can also be measured for these adult amphipods.

Results Interpretation

Grain Size

Hyallela azteca tolerate a wide range in sediment grain size and organic matter. During development of this test by the EPA,USFWS, and during the round robin assays, no significant correlations were observed between the survival, growth, or reproduction of *H. azteca* and the physical characteristics of the sediment (grain size ranging from predominantly silt to predominantly sand), TOC (ranging from 0.3 to 9.6%), water content (ranging from 19 to 81%; Ingersoll et al. 1998). Additionally, no significant correlations were observed between these biological endpoints and water quality characteristics (i.e., hardness, alkalinity, ammonia) of pore water or overlying water in the sediments evaluated by Ingersoll et al. (1998). Weak trends were observed between reproduction of amphipod percent clay, percent silt, and percent sand. The weak relationship between the sediment grain size and reproduction in that study may have been due to the fact that samples with higher amounts of sand also had higher concentrations of organic contaminants compared to other samples evaluated in Ingersoll et al. (1998).

Influence of Indigenous Organisms

Survival of *H. azteca* in 28-d tests was not reduced in the presence of oligochaetes in sediment samples (Reynoldson et al. 1994). However, growth of amphipods was

reduced when high numbers of oligochaetes were placed in a sample. Therefore, it is important to determine the number and biomass of indigenous organisms in field-collected sediments in order to better interpret growth data (Reynoldson et al. 1994). Furthermore, presence of predators may also influence response of test organisms in sediment (Ingersoll and Nelson 1990).

Number of Replicates

Although 8 replicates are recommended for evaluating reproduction in the long-term sediment tests with *H. azteca*, there may be instances where either additional or fewer replicates might be evaluated to monitor reproduction (Ingersoll et al. 1998). For example, Kubitz et al. (1996) recommended a two step process for assessing growth in sediment tests with *H. azteca*. Using this process, a limited number of replicates would be tested in a screening step. Samples identified as possibly affecting reproduction could then be tested in a confirmatory step with additional replicates. This two-step analysis conserves laboratory resources and increases statistical power when needed to identify sublethal effects. A similar approach could be applied to evaluate reproductive effects of contaminants in sediment where a limited number of replicates could be initially tested to evaluate potential effects. Samples identified as possibly toxic based on reproduction could then be reevaluated using an increased number of replicates. It should be noted that results of reproduction have been mixed, and the value of this endpoint is still under consideration.

Table 1. Test conditions for conducting a whole-life cycle assay with *Hyalella azteca*.

Parameter	Conditions
1. Test Type	Whole-sediment toxicity test with renewal of overlying water
2. Temperature	23±1°C
3. Illuminance	About 500 to 1000 lux
4. Photoperiod	16L:8D
5. Test chamber	300 mL high-form lipless beakers
6. Sediment volume	100 mL
7. Overlying water volume	175 mL in the sediment exposure from day 0 to day 28 and 175-275 mL in the water only exposure from day 28 to day 42
8. Age of organisms	7 to 8 d old at the start of the test
9. Number of organisms/chamber	10
10. Number of replicate chambers/treatment	12 (4 for 28 d survival and growth and 8 for 35 and 42 d survival, growth, and reproduction).
11. Feeding	YCT food, fed 1.0 mL (1800 mg/L stock) daily to each test chamber
12. Aeration	None unless DO in overlying water drops below 2.5 mg/L
13. Overlying water	Preferably culture water. Well water, surface water, or site water may also be used.
14. Test chamber cleaning	Occasionally the outside of the screen may be brushed gently with a toothbrush.
15. Overlying water quality	Hardness, alkalinity, conductivity, and ammonia at the beginning and end of a test (day 0 and day 28). Temperature daily. Conductivity weekly. Dissolved oxygen (DO) and pH three times/week. Concentrations of DO should be measured more often if DO drops more than 1 mg/L since the previous measurement.
16. Test duration	42 d
17. Endpoints	28 day survival and growth; 35 day survival and reproduction, 42 day survival, growth, reproduction, and number of adult males and females.
18. Test acceptability	Minimum mean control survival of 80% on day 28 and suggested performance-based developed following round-robin testing

Table 2. General activity schedule for conducting a whole-life cycle assay with *Hyalella azteca*.

Day	Activity
-8	Isolate several hundred adult amphipods from culture and place into breeding chambers.
-7	Remove adults from breeding chambers and isolate <24h old amphipods
-6 to -1	Feed and observe isolated amphipods.
-1	Add sediment into each test chamber, place chambers into exposure system, and start renewing overlying water
0	Measure total water quality (pH, temperature, DO, hardness, alkalinity, conductivity, ammonia). Transfer ten 7 to 8 d old amphipods into each test chamber. Release organisms under the surface of the water. Add 1.0 mL of YCT into each test chamber.
1 to 27	Add 1.0 mL of YCT to each test beaker. Measure temperature daily, conductivity weekly, and DO and pH three times/wk. Observe and record behavior of test organisms.
28	Measure temperature, DO, pH, hardness, alkalinity, conductivity and ammonia. End the sediment exposure portion of the test by collecting the amphipods with a #40 mesh sieve (425 μ m mesh). Use four replicates for growth measurements: count survivors and preserve organisms in sugar formalin for growth measurements. Eight replicates for reproduction measurements: place survivors in individual replicate water-only beakers and add 1.0 mL of YCT to each test beaker/d and 2 volume additions/d of overlying water.
29 to 35	Feed daily. Measure temperature daily, conductivity weekly, DO and pH three times a week. Measure hardness and alkalinity weekly. Observe behavior of test organisms.
35	Record the number of surviving adults and remove offspring. Return adults to their original individual beakers and add food.
36 to 41	Feed daily. Measure temperature daily, conductivity weekly, DO and pH three times a week. Measure hardness and alkalinity weekly. Observe behavior of test organisms.
41	Same as day 1. Measure total water quality (pH, temperature, DI, hardness, alkalinity, conductivity, ammonia).
42	Record the number of surviving adults and offspring. Surviving adult amphipods on day 42 are preserved in sugar formalin solution. The number of adult males in each beaker is determined from this archived sample. This information is used to calculate the number of young produced per female per replicate from day 28 to day 42.

Table 3. Test acceptability requirements for a whole-life cycle assay with *Hyalella azteca*.

C. The following performance criteria for the whole life stage test with <i>H. azteca</i> have been recommended by the EPA:	
1.	Age of <i>H. azteca</i> at the start of the test should be 7 to 8 d old. Starting a test with substantially younger or older organisms may compromise the reproductive endpoint.
2.	Average survival of <i>H. azteca</i> in the control sediment on day 28 should be greater than or equal to 80%.
3.	Laboratories participating in round-robin testing reported after 28 d sediment exposures in a control sediment (West Bearskin Lake), survival >80% for >88% of the labs; length > 3.2 mm/individual for > 83% of the labs; and weight > 0.15 mg/individual for 67% of the labs. Reproduction from day 28 to day 42 was >2 young/female for 63% of the labs. Reproduction was more variable within and among laboratories; hence, more replicates might be needed to establish statistical differences among treatments with this endpoint.
4.	Hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the sediment exposure.
B. Additional Requirements	
1.	All organisms in a test must be from the same source.
2.	Negative-control sediment must be included in a test.
3.	Test organisms should be cultured and tested at 23°C.
4.	The mean of the daily test temperature must be 23±1°C. The instantaneous temperature must be 23±3°C.

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