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The Potential of a Sediment Avoidance Test for Assessment of Hazardous Waste Sites

Roger B. Yeardley Jr.

(Retired) U.S. Environmental Protection Agency, Cincinnati, OH, USA
Email: yeardleyroger@gmail.com

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Abstract

Based on the success of an earthworm soil avoidance test and the value of a similar fast, inexpensive tool for assessment of sediment at the large number of contaminated sites worldwide, experiments were undertaken to explore the development of a sediment avoidance test. Results of mobility tests with only control sediments were a key factor in choosing *Hyalella* for this test from a list of 3 candidate organisms with a history of use in standardized ecotoxicology tests: *Hyalella azteca*, *Lumbriculus variegatus*, and *Chironomus dilutus*. In these tests *Chironomus* individuals generally stayed on the side of chambers where they were deployed ($p = 0.0019$). *Hyalella* was found in relatively equal numbers on each side of the chambers ($p = 0.346$), while Lumbriculus was inconsistent in this regard. *Hyalella* avoided contaminated sediments, both in tests with field site sediments from multiple locations containing a variety of contaminants (metals PCBs, PAHs, dioxins/furans) and tests with sediments spiked with the ubiquitous, persistent contaminants PCBs and PAHs. There was evidence of increased sensitivity of the avoidance test, as well as a correlation between avoidance and the more standardized endpoints of growth and survival. *Hyalella* avoided sediments with significant mortality, and to some which showed little mortality in acute tests. test showing more sensi. As part of test development, the effects of other behaviors on test results and interpretation, including phototaxis and thigmotaxis, were explored. This test can be conducted with inexpensive, easily available equipment and materials, making it accessible to a wide range of groups and individuals. Therefore, this 24-hour *Hyalella* sediment avoidance test has good potential for use in assessment of contaminated sediments.

Keywords

Avoidance, Sediment, Contaminated, *Hyalella*, Behavior, Ecotoxicity

1. Introduction

There is great need for assessment and remediation of contaminated sites across the world. A recent study by Zhang *et al.* [1] concluded that 1,163,977 contaminated sites exist worldwide, across 131 countries/regions which pose threats to ecosystems and human health. The water and sediment in waterbodies are the ultimate sink for most contaminants, even ones that begin as contaminants in soil and air. In the U.S. over 78 million people live within 3 miles of 1881 Superfund hazardous waste sites [2]. In the U.S. over 30 million people live in in the Great Lakes Basin [3]. Many of these people live near Great Lakes Area of Concern (AOC) sites [4], monitored, assessed, and remediated by the U.S, Environmental Protection Agency and its partners, and where the environmental degradation often involves contaminated sediments.

A simple soil avoidance test with the earthworm *Eisenia fetida*, since its development [5], has become an international standard [6] used to assess contaminated soils. Perhaps a similar sediment avoidance test could help feel a need for tools to assess contaminated sediment sites. Avoidance tests are typically faster and less expensive than acute and many standard toxicity tests, and also useful as screening tools. Avoidance tests are a type of behavioral ecotoxicity test with ecological relevance that permit assessment of contaminant concentration thresholds affecting macroinvertebrate community dynamics. Avoidance is ecologically relevant in that it is a behavioral endpoint which can affect the structure and function of sediment macroinvertebrate communities. Since it is often observed at sublethal levels of contaminants, it can give information beyond what can be obtained with acute tests. There is also evidence of soil avoidance tests being more sensitive to a variety of contaminants than acute and reproductive tests [1] [7]-[13].

Researchers have observed avoidance of contaminated sediments by a variety of freshwater and saltwater organisms. Szczybelski *et al.* [14] found that both freshwater amphipod *Gammarus pulex* and marine amphipod *Gammarus locusta* avoided oil-spiked sediments. Kravitz *et al.* [15] showed that the amphipod *Euhaustorius estuarius* avoided PAH-contaminated sediments, and concluded avoidance to be a more sensitive endpoint than mortality. The amphipod, *Monoporeia affinis*, avoided sediments contaminated with zinc pyrithione for Wiklund *et al.* [16]. Keilty *et al.* [17] observed avoidance by two freshwater oligochaetes of sediments contaminated with sublethal levels with endrin, where coming to the sediment surface (vs. burrowing) was considered avoidance. West and Ankley [18] used a unique chamber design to assess 12 sediments, using *Lumbriculus variegatus* as their test organism. They found that *L. variegatus* avoided many of these sediments, both field and spiked, to which the organism showed no significant growth or survival effects. Ward *et al.* [19] observed avoidance of contaminated sediments collected from estuarine sites by amphipod, copepod, and snail species. They concluded that avoidance was related to sediment toxicity. Test organisms avoided contaminated sediments in as little as 6 hours. In experiments by Araújo *et al.* [20], the snail *Hydrobia ulvae* avoided contaminated sediments.

The candidate organisms that were assessed for potential use in a sediment avoidance test were *Chironomus dilutus* larvae, the amphipod *Hyalella azteca*, and the oligochaete *Lumbriculus variegatus*. These organisms are used in standard EPA methods for sediment toxicity and bioaccumulation; EPA Methods 100.1 [21], 100.2 [22], and 100.3 [23]: Their history of use means that these organisms are known to be sensitive to contaminants and that there are established culture methods available. These organisms are also widely found in the environment, and therefore ecologically relevant.

The first phase of this study was selecting one of these 3 candidates as the best to use in sediment avoidance test development. Two key criteria of organisms' suitability for use in avoidance tests were sensitivity to contaminants and mobility, or tendency to move through/explore the sediment within the chambers. They must be able to sense toxic chemicals and move away from them. Other criteria worth considering are ease of culturing and handling, and other behaviors that might interfere with or obscure contaminant avoidance behavior. One advantage of using standard test organisms is that they have an established history of sensitivity to contaminants, *i.e.* already meet one of the two key criteria. A series of tests were devised and run to assess the movement of organisms within the test chambers containing only control sediment as a measure of mobility. Though the aquatic oligochaete *Lumbriculus* was an early favorite due to previous success with using the terrestrial oligochaete *Eisenia fetida* in soil avoidance tests [5] and the fact that aquatic oligochaetes are in contact with sediment both internally and externally, based largely upon the results of mobility test results, *Hyalella* became a prime candidate. Test conditions were varied, especially light vs dark and test duration in order to design an avoidance test in which the organisms' other behaviors (e.g., mating, predator avoidance, phototaxis, thigmotaxis) did not interfere with or obscure an avoidance-of-chemical-contaminants response. Light and dark and test duration were also varied in an attempt to remedy inconsistencies observed in tests with Lumbriculus regarding mobility and independent movement. Once we had chosen *Hyalella* as the best candidate organism, we ran tests with this organism on sediments from contaminated sites as well as sediments spiked with the ubiquitous sediment contaminants, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs).

2. Materials and Methods

2.1. Test Chamber Construction and Modifications

Test chambers were the same 150 × 75 mm glass crystallizing dishes used in the earthworm soil avoidance test [5] [6]. There were two versions, one for testing with *Chironomus* and *Lumbriculus* (**Figure 1(a)**), and another for use with *Hyalella azteca* (**Figure 1(b)**). Chambers for *Chironomus* and *Lumbriculus* were dishes with only slight modification; a midline drawn onto the outside of the chamber and a separate divider for use in test setup and takedown. Modifications, which involved installing permanent slots/runners and specialized dividers, were made to accommodate the use of *Hyalella*. For these modifications, 1/4" (6 mm) Plexiglas was used to fabricate the dividers and the runners used to hold the dividers in place. The runners were approximately 6 mm × 6 mm × 6.5 cm and held in place with aquarium-safe silicone compound. Importantly, silicone is also added between the outside edges of the runners and the chamber walls. Having a smooth surface of silicone filling these spaces, keeps the *Hyalella* from squeezing into and hiding in spaces, which can otherwise occur between the runner and the wall. The bottom dividers, that separate the control and test sediments, were 20 mm high in these tests. The bottom dividers can be held in place with silicone, but this is not necessary. Researchers can customize the height of these to accommodate different volumes of sediment, and/or if they want to adjust the height to be flush with the sediments on either side. The upper dividers, which are put in at the end of the exposure period and held between the runners at the midlines of the chambers, were 5 cm tall (and 6 mm wide).

Other modifications to chambers and/or protocols included covering half of the chambers with black plastic for phototaxis experiments (Figure 2(a)), removing a central core (Figure 2(b)) to examine thigmotactic/edge effects.

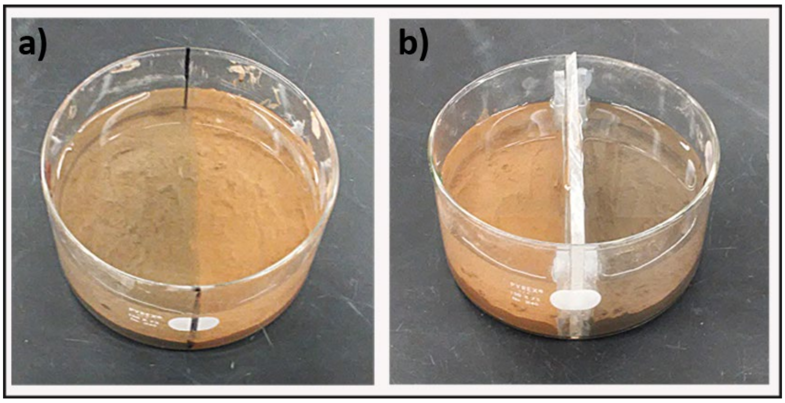

Figure 1. Two versions of the test chambers used. (a) Version used in tests with *Lumbriculus variegatus* and *Chironomus dilutes*; (b) Modified version, with tracks for midline divider added, used in tests with *Hyalella azteca*.

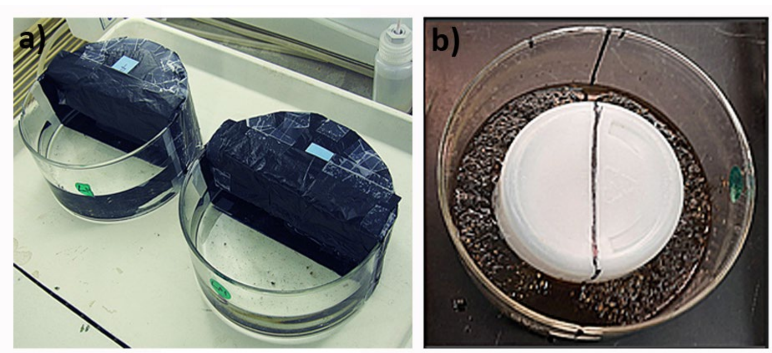

Figure 2. Chamber and protocol modifications for special tests: (a) chambers modified for phototaxis experiments (without sediment present), and (b) sediment core for thigmotaxis experiments.

2.2. Avoidance Test Conditions and Protocols

2.2.1. Test Protocols Summary

Tests were run for 24 or 48 hours, in an incubator at 22˚C (±1˚C). A 24-hour duration was settled upon early in the process as part of the protocols for the *Hyalella* sediment avoidance test. For all tests four replicate test chambers were used per test, with 20 organisms per test chamber. Tests were conducted under continuous light, with measured light levels in the range of 1020 - 1220 lux. 125 ml of control sediment was added to one side of the chamber, 125 ml of test sediment to the other side, and 500 ml of distilled water added as overlying water. Replicate sediment samples added to the 4 chambers, whether control or test sediments, were taken from one homogenized sample of that sediment. Large, mature organisms of all 3 species were used in all tests. *Hyalella* were from cultures 3 - 5 months old, most in the 3 - 4 months range.

2.2.2. Control Sediments Used

A variety of control sediments were used over the span of this study. Some control sediments were collected from natural sites. One control sediment used in some early testing of contaminated sediments from the Bunker Hill Superfund/Lane Marsh site, was sediment from West Bearskin Lake near Grand Marais, Minnesota which was supplied by EPA colleagues in Duluth, MN and used in previous experiments [18] [24]. Another natural site-collected sediment was from a non-contaminated wetland near Lane Marsh. Commercial topsoils were used to make control sediments for a number of tests. These were obtained from Home Depot or Lowe's in 40 lb. bags or acquired from a local landscaping company (spiked-sediment tests). Commercial topsoils were chosen which did not have any fertilizer added. The control sediments used in the different experiments and some key characteristics are listed in **Table 1**. Percent organic content and percentages of sand, silt, and clay were measured according to USEPA SOP #3953-1 [25].

Control sediments from commercially available topsoils and some site sediments contained a significant amount of large coarse particulate organic matter (CPOM). To have sediments of more uniform consistency and to make finding organisms easier during test takedown, this CPOM was removed by sieving through stacked sieves: 2 mm (#10 ASTM), 500 µm (#35 ASTM), 63 µm (#230 ASTM), top to bottom (**Figure 3**). Topsoil was placed on the top (2 mm) sieve, and the soil was rinsed from above until only the large woody debris remained. The large debris on the 2 mm sieve was disposed of, and what remained on the 500 µm and 63 µm sieves was mixed together and hydrated with distilled water to produce the control sediment. This sieving process was also used on some site sediments, like those from Lane Marsh, which contained a lot of plant matter, leaves and stems from wetland plants.

2.2.3. Test Setup

Approximately 125 ml each of the control and test sediments were measured into separate 150 ml beakers. With dividers in place, scoop the sediments out of the beakers and into control and test sides of the chambers. For sediments of a more solid consistency, a spoon was used to spread the sediments out evenly on each side. Then the dividers were removed and a spoon used to push the sediments together and remove the small gap along the midline where the divider was. For more "watery" sediments spreading with a spoon may not be necessary, and a gentle tapping of the chambers (on a not-too hard surface like a lab notebook) after removal of the divider will bring the sediments into contact at the midline. Make sure each side of the chamber is labeled to distinguish it as containing test or control sediment. Round green and red "dot" stickers were attached on the control and test sides respectively, for this purpose. Loosely place semicircular thin plastic covers on top of the sediment on each side of the chamber to prevent dispersing the sediment into the water column. Then, slowly pour 500 ml of distilled water into each chamber. Put the chambers into a lighted incubator at 22˚C and allow equilibration of water and sediment for 2 - 3 hours. Then, from your culture of *Hyalella*, count out 20 adult individuals into each of 4 small beakers or cups with distilled water, avoiding "paired" organisms. Pour the 20 individuals contained in each beaker into the middle (or, if you prefer, the test side) of each chamber.

Table 1. Control sediments used for avoidance test development experiments. Same = same sediment used on control and test sides of chambers. * = natural site sediment.

			Control Sediment				Test Sediment			
Control Soil/Sediment	**Avoidance Experiment**	**Brand/Source**	**% Organic**	**% Sand**	**% Silt**	**% Clay**	**% Organic**	**% Sand**	**% Silt**	**% Clay**
Bearskin Lake*	Lane Marsh 3 (test1)	Grand Marais, MN	1.6%	41.7%	56.7%	1.7%	20.0%	0.0%	98.5%	1.5%
Lane Marsh Reference*	Lane Marsh 1	Coeur d'Alene, ID	11.8%	0.0%	96.0%	4.0%	17.5%	0.0%	95.2%	4.8%
Topsoil 1	Lane Marsh 3 (test 2)	Timberline brand	9.8%	54.1%	40.5%	5.4%	20.0%	0.0%	98.5%	1.5%
Topsoil 1	Iron King Mine (50%)	Timberline brand	9.8%	54.1%	40.5%	5.4%	2.38%	22.0%	76.0%	2.0%
Topsoil 2	Mobility Tests	Organic Valley brand	15.5%	37.1%	60.0%	2.9%	Same	Same	Same	Same
Topsoil 3	PAH-Spiked	Bzak Landscaping	6.1%	32.4%	65.3%	2.4%	Same	Same	Same	Same
Topsoil 3	PCB-Spiked	Bzak Landscaping	6.1%	32.4%	65.3%	2.4%	Same	Same	Same	Same
Topsoil 4	Erie Pier Ponds	Organic Valley brand	10.3%	62.5%	35.4%	2.1%	14.7%	17.0%	83.0%	0.0%

2.2.4. Test Takedown

After 24 hours in the incubator, the first step in the takedown of tests with *Hyalella*, is to quickly place the Plexiglas dividers in the tracks constructed for them at the midlines of the test chambers. Do this first, before any other handling of the test chambers. This is done to avoid disturbing the organisms and causing them to swim and relocate to different positions in the chambers. Check the cups with removed water for organisms that may have been accidentally removed. Use a large pipette or syringe to remove 50 - 100 ml of overlying water for water chemistry pH, conductivity, dissolved oxygen and temperature measurement. To remove and count the organisms, one method is to process all the water and sediment from each side of the chamber through a 500 μm sieve and run water through the sieve until a much-reduced amount of sediment and the organisms remain. Then rinse the sieve contents into large, flat containers and pipette off and count the organisms. Glass casserole dishes (9" × 13") were used for this step. Over time, a preferred alternate method was adopted. First, the overlying water and organisms were pipetted off into the labelled casserole dishes (one for each side of the chamber) using a large pipette or "turkey baster". Once most of the water has been removed, the remaining *Hyalella* can be removed using smaller disposable pipettes. Jiggling the chambers a little during this process is useful to dislodge amphipods from the top layer of sediment and get them swimming in the overlying water, making them more accessible to pipetting. Pipette the organisms from the casserole dishes into small beakers or plastic cups and count the number found on each side. If the total number is less than 20, then sieve the sediments to search for the missing organisms. For testing with *Lumbriculus*, you can first pipette/remove some organisms from the sediment surface. But, since these organisms are not epibenthic like *Hyalella*, very rarely will they all be on the surface, so sieving is also necessary. The results at takedown of numbers of organisms found on the control and test sediment sides are analyzed by 2-tailed t-tests (unpaired). Avoidance is considered to have occurred if more organisms were on the control side and $p < 0.05$. Paired t-tests were not used, because individuals of a successful test candidate species would be expected to move independently within the chambers.

Figure 3. Stacked sieves were used to produce control sediments from commercial topsoil, and to process site soil with significant amounts of large organic matter.

2.2.5. Chemical/Physical Measurements

The following chemical and physical measurements were made on samples of the overlying water, collected at the beginning and end of each test: conductivity, pH, DO, and temperature. The average values at the start and end of all experiments are listed in Table 2. Conductivity and pH were measured using the Oakton Series 11 conductivity and pH meters. Do was measured with a YSI Pro DO meter, and water temperature checks done with Digi-Sense digital thermometer. Samples from the sediments and topsoils were analyzed for organic content by an ash-free dry mass AFDM method, and sand, silt, and clay by a gravimetric method [25].

Table 2. Chemical and physical measurements from each test. Values are averages from 4 replicate chambers. # Diff. = number of tests where there was a significant difference. Diff.? = was there a significant difference? (where there was only one test). Average values at test start and end were considered to be different if the results of paired t-tests were <0.05.

MOBILITY EXPERIMENTS								
	Hyalella							
	Cond. (uS)		pH		DO (mg/L)		Temp. (˚C)	
	Start	End	Start	End	Start	End	Start	End
	419	473	7.19	6.71	8.3	6.2	20.5	22.9
	679	736	7.19	7.23	6.5	5.8	21.2	21.2
	674	909	7.72	6.96	7.8	3.4	24.6	23.8
# Diff. >>	1		2		3		2	
	Lumbriculus							
	Cond. (uS)		pH		DO (mg/L)		Temp. (˚C)	
	Start	End	Start	End	Start	End	Start	End
	699	1237	7.31	7.82	5.4	7.3	24.1	23.5
	534	801	7.1	7.44	8.1	2.3	22.8	23
	643	1243	7.97	7.75	8.7	2.5	18.9	21.2
# Diff. >>	3		3		3		1	
	Chironomus							
	Cond. (uS)		pH		DO (mg/L)		Temp. (˚C)	
	Start	End	Start	End	Start	End	Start	End
	350	484	7.01	6.6	8.1	6	21.6	21.2
	339	648	6.38	6.73	8.4	7.4	21.1	21.4
	638	1058	7.46	7.54	5.9	4.7	24.6	24.1
# Diff. >>	2		2		3		3	
SPIKED SEDIMENT EXPERIMENTS								
	PCBs							
	Cond. (uS)		pH		DO (mg/L)		Temp. (˚C)	
	Start	End	Start	End	Start	End	Start	End
	177	346	5.81	5.73	5.4	6.6	20.7	20.2

Continued

	174	347	5.8	5.88	5.3	5.3	21.7	21.8
	129	305	5.83	5.95	6	6.9	19.9	20
# Diff. >>	**3**		**1**		**2**		**1**	
	PAHs							
	Cond. (uS)		**pH**		**DO (mg/L)**		**Temp. (˚C)**	
	Start	**End**	**Start**	**End**	**Start**	**End**	**Start**	**End**
	159	356	5.68	5.82	7.4	4.7	21.1	21
	184	303	5.75	5.83	5.7	5.4	21.6	20.5
	159	320	5.88	5.94	6.6	6.9	20.7	20.5
# Diff. >>	**3**		**0**		**1**		**2**	
SITE SEDIMENT EXPERIMENTS								
	LM-1							
	Cond. (uS)		**pH**		**DO (mg/L)**		**Temp. (˚C)**	
	Start	**1**	**Start**	**End**	**Start**	**End**	**Start**	**End**
	258	276	6.07	5.63	6.9	6.7	22.5	22.5
Diff. ? >>	**N**		**Y**		**Y**		**N**	
	LM-3							
	Cond. (uS)		**pH**		**DO (mg/L)**		**Temp. (˚C)**	
	Start	**1**	**Start**	**End**	**Start**	**End**	**Start**	**End**
	435	402	6.59	6.34	6.2	6.7	23.1	23.4
Diff. ? >>	**N**		**N**		**Y**		**Y**	
	IKM50							
	Cond. (uS)		**pH**		**DO (mg/L)**		**Temp. (˚C)**	
	Start	**End**	**Start**	**End**	**Start**	**End**	**Start**	**End**
	1234	1408	6.42	6.54	3.2	4.5	22.3	23.6
Diff. ? >>	**Y**		**Y**		**Y**		**Y**	
	EPP-04							
	Cond. (uS)		**pH**		**DO (mg/L)**		**Temp. (˚C)**	
	Start	**End**	**Start**	**End**	**Start**	**End**	**Start**	**End**
	136	407	5.91	6.23	7.6	7.2	20.8	20.4
Diff. ? >>	**Y**		**Y**		**Y**		**Y**	

2.3. Growth and Survival Tests

Acute and growth tests with *Hyalella* were 7-Day, reduced volume, with constant light, according to SOP-1361-0 [26], with some adjustments for the tests with PAH- and PCB-spiked sediments. To make test conditions similar to those used in the avoidance tests with the same spiked sediments, the incubator was set to 22˚C instead of the 25˚C specified in the SOP, and distilled water was used instead of moderately hard reconstituted water [27].

2.4. Mobility Experiments Protocols

Mobility tests were conducted with control sediments made from commercially available topsoil (which involved sieving as per section 2.1) placed on both sides (125 ml per side) of the test chambers. At the start of tests 20 organisms were deployed into the water on one side, referred to here as the "deployed side", with the divider in place. The divider was left in place for approximately 5 minutes after deployment of organisms and then removed to start the 24-hour tests. Sediments used for the mobility tests (both control and "test" sediments) were made from commercially available Organic Valley brand topsoil (**Table 1**).

2.5. Site Sediment Experiments Protocols

The concentrations and mix of contaminants of the specific sediment samples used for ecotoxicity tests were not measured. The site sediment experiments are proof-of-concept. Contamination status was inferred from site history rather than direct analysis, and comparisons were made of the results of avoidance tests to those of growth and survival tests. All sediments used in avoidance and acute tests, except that from Iron King Mine, represented homogenizations of multiple grab samples collected at a specific station.

Sediments from stations LM-1 and LM-3 in Lane Marsh, a site located adjacent to the Lower Coeur d'Alene River and part of the Bunker Hill Superfund site [28] in Coeur d'Alene Idaho, were tested. Bunker Hill is contaminated from decades of mining and refining lead (Pb). Samples of sediment from station LM-3 were collected on 10/27/17. Grab samples of control sediments from an uncontaminated wetland near Lane Marsh as well as sediments from station LM-1, were collected on 5/28/18 (**Table 1**).

Soil samples from the tailings pile of the smelter at the Iron King Mine (IKM) Superfund site in Arizona [29], contaminated with arsenic (As), lead (Pb), iron (Fe) and other heavy metals, were mixed with commercial topsoil, to make another metals-contaminated sediment in addition to those from Lane Marsh. This Superfund site was the source of the mine waste-derived fertilizer, Ironite [30]. The sample of IKM soil used in this study was taken from a larger supply of dry soil stored at the USEPA, Cincinnati from a previous study in 2006 [30]. Control sediments used for this test were made from commercially available Timberline topsoil (**Table 1**).

Grab samples of sediment from station EPP-04 were collected on 7/25/23 from the Ponds Behind Erie Pier site in downtown Duluth, MN. This site is part of the St. Louis River AOC [31]. The sediment in these ponds is contaminated with cadmium, chromium, copper, lead, mercury, nickel, zinc, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins/furans [32]. However, the sample was collected was post-remediation, and thus the levels of these contaminants were presumably significantly reduced. Control sediments used for this test were made from commercially available Organic Valley topsoil (**Table 1**).

2.6. Spiked Sediment Experiments Protocols

2.6.1. Production of Spike Solutions

For each contaminant type, neat compounds were mixed with acetone. For PAHs, 32 vials (each vial containing 10 mg of a particular PAH; 16 priority PAHs, 2 vials per PAH) were used. 16 × 10 × 2 = 320 mg of total PAHs (20 mg of each of the 16 PAHs) (**Table 1**). For some PAHs, the neat compounds did not dissolve in acetone. For those PAHs, 10:1 Acetone: dichloromethane (DCM) was used (400 ml acetone and 44 ml DCM). For PCBs, 3 vials, each containing 50 mg Aroclor 1254, were used. For PAHs and PCBs acetone and neat compounds were mixed by vortexing. Vials were rinsed 2 times, and these rinses added to the total volume (PAHs = 444 ml; PCBs = 400 ml). PAH stock conc: 320 mg PAHs/(444/1000) = 720 mg/L in solvent. PCB stock conc: 150 mg PCB/(400/1000) = 375 mg/L in solvent. Control sediments and spiked test sediments used for these tests were made from topsoil purchased from Bzak Landscaping in Cincinnati, OH (**Table 1**).

2.6.2. Mixing Contaminants with Soil

Soils and spike solutions were mixed together with a Hobart commercial food mixer. For PAHs, 320 mg of total PAHs were mixed into 3 Kg soil (**Table 3**). For PCBs, 150 mg of Aroclor 1254 were mixed into 3 Kg soil. A mixer was first cleaned and decontaminated (soap washed, cleaned metal parts with acetone). Approximately half (~1400 ml) of the soil was added to the mixer first. Around 400 ml of PAH or PCB spike solution and remaining half of the soil was added slowly in small batches. Mixing was done for 60 minutes, in two 30-minute segments with a break in between. Once mixed, the spiked soil mixture was added to aluminum foil trays and dried for 24 - 48 hr. in a clean fume hood until the mixture was completely dry, and all the solvent evaporated before storing in clean 2-gallon buckets. Test sediments were produced by hydrating the dry spiked soils to saturation with distilled water. PCB spiked soil conc: 150/3 = 50 mg/Kg nominal concentration (total PCB) on a dry soil volume basis. PAH spiked soil conc: 320/3 = 107 mg/Kg nominal concentration (total PAH) on a dry soil volume basis.

Table 3. Components in the PAH stock used to spike sediments.

PAH Compound	Amt. Added	PAH Compound	Amt. Added
Acenaphthene	20 mg	Dibenz(a,h)anthracene	20 mg
Anthracene	20 mg	Fluoranthene	20 mg
Benz (a) anthracene	20 mg	Fluorene	20 mg
Benz [a] pyrene	20 mg	Indeno(1,2,3-cd)pyrene	20 mg
Benzo (b) fluoranthene	20 mg	Naphthalene	20 mg
Benzo(g,h,i)perylene	20 mg	Phenanthrene	20 mg
Benzo (k) fluoranthene	20 mg	Pyrene	20 mg
Chrysene	20 mg	Total PAHs	**320 mg**

3. Results

3.1. Mobility Experiments Results

At the end of mobility tests there were approximately equal average percentages (3 tests) of *Hyalella azteca* and *Lumbriculus variegatus* on both sides of chambers, Only in the tests with *Chironomus dilutus* larvae were there consistently higher numbers at test end still on the side on which they were introduced/deployed (Table 4, Figure 4). *Lumbriculus* mobility results were the most inconsistent with respect to overall results, with more of the organisms on the deployed side in two experiments (78%, 58%) and more on the undeployed side in the other (61%), as well as in terms of variability (standard deviations, Figure 4).

In the absence of test sediment, a high frequency of high numbers on one side of chambers suggests a lack of independent movement. A more detailed look at the results of the mobility tests supports the assumption that *Hyalella* are moving independently, but for *Lumbriculus* they do not give support. Looking at the frequency at which high numbers were seen on one side of the chambers, *Hyalella* showed ≥75% of individuals (≥15 individuals) on one side in 3 of the 12 chambers (3 tests × 4 chambers) or 25% frequency, while *Lumbriculus* had ≥75% of individuals (≥14 individuals) in 6 of 12 chambers for a 50% frequency. If we expand the cutoff to ≥14 individuals (≥70%), *Lumbriculus* increases to 75% (9/12 chambers) while *Hyalella* stays at 25%.

Additional control tests (tests with only control sediment in the chambers) run with *Lumbriculus* under different conditions to try to address the inconsistency issue, instead reinforced that there was an issue. For example, in 2 control tests with constant light but a 48-hour duration, there were ≥75% of individuals in 50% (2/4) and 75% (3/4) of the chambers. In a 48-hour test under dark conditions, ≥75% of Lumbriculus individuals were found on one side in 100% (4/4) of the chambers. In two 24-hours tests in the dark, this condition was met in 25% (1/4) and 75% (3/4) of chambers.

Average recovery from 9 mobility tests with 3 organisms was 97.7%.

Table 4. Results of the mobility experiments, where a p value of >0.05 indicates that there were not more organisms on either side of the chambers.

	p values			
Organism	Test 1	Test 2	Test 3	Average
Hyalella azteca	0.101	0.556	0.381	0.346
Chironomus dilutus	0.000023	1.19E−09	0.00566	0.0019
Lumbriculus variegatus	6.77001E−07	0.226	0.648	0.291

3.2. Site Sediment Experiments Results

The results of 24-hour avoidance tests with *Hyalella* were compared with those of 7-day growth and survival tests (Table 4), using contaminated sediments collected from a Great Lakes Area of Concern (St. Louis River), sediment collected from two stations at one Superfund Site (Lane Marsh/Bunker Hill), and sediment made from soil from another Superfund Site (Iron King Mine) (Figure 5, Table 5). Sediments from the two stations with no significant mortality (LM-3, EPP-04), one of which also showed no weight loss (LM-3), also showed no avoidance of the test sediment. For station LM-3 there were actually more organisms in the test soil than in the control soil at test end (Figure 5, Table 5). Bearskin Lake sediment was the control sediment used for that test. In a second test with topsoil as the control sediment (Table 1), most of the organisms (61%) again ended up in the LM-3 soil (p = 0.004). *Hyalella* avoided sediment from the station (LM-1) which had zero percent survival in the acute test (Table 5). Sediment produced from 50% soil from site/station IKM and 50% control soil (IKM50 in Table 5, Figure 5), which showed reduced growth but not mortality, was avoided. For each test sediment, the same control sediment was used for acute and avoidance tests. Average recovery of *Hyalella* from the 5 avoidance tests with site sediments was 95.3%.

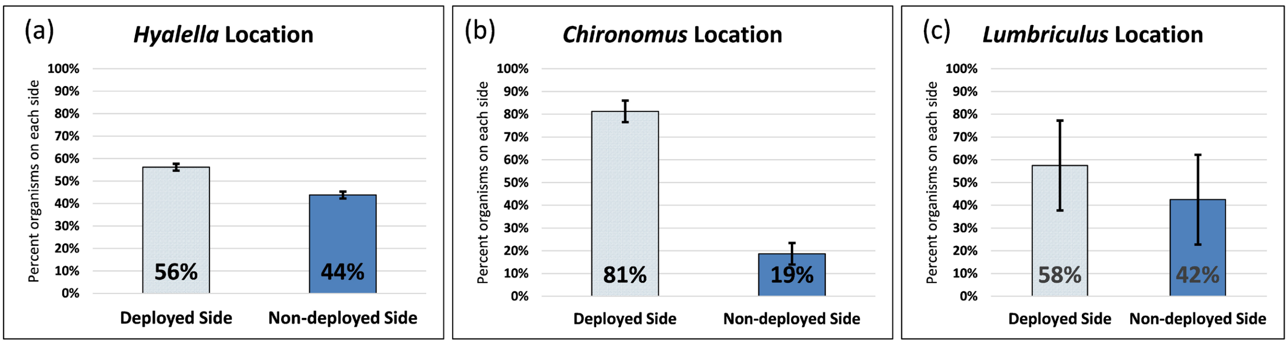

Figure 4. Mobility experiments—did organisms remain on the side of chambers where they were deployed or move to occupy both sides of chambers equally? (a) *Hyalella* spread out fairly evenly on both sides of the chambers, (b) most of the *Chironomus* larvae largely stayed on the side on which they were deployed, and (c) *Lumbriculus* results were inconsistent. Error bars represent the average standard deviation of 3 tests.

Table 5. Correlations between growth/survival and sediment avoidance by *Hyalella* for sediment from 4 stations/3 sites. NA—Weight loss not measured. NA**—Weight loss can't be calculated because all organisms died in the acute test. N*—though the p for avoidance was 0.05, there were significantly more organisms on the LM-3 side (Figure 5) at test end.

Site Sediment	p value -Acute	% Survival	p value-Weight Loss	p value-Avoidance	Mortality?	Weight Loss?	Avoidance?
LM-1	0.012	0%	NA**	0.0014	Y	NA**	Y
LM-3	0.39	95%	0.164	0.0019	N	N	N*
IKM	0.67	95%	0.0036	0.00002	N	Y	Y
EPP-04	1.000	95%	NA	0.097	N	NA	N

3.3. Spiked Sediment Experiments Results

Hyalella avoided both the PCB (conc. 50 mg/Kg) and PAH-spiked (conc. 107 mg/Kg) sediments. In a series of 3 tests, the majority of organisms were found in the control sediment at the end of tests (Figure 6). The average p values (2-tailed) from 3 tests were 0.005 for PCBs and 0.02 for PAHs. Seven-day acute tests showed no significant difference in survival between the contaminated and control sediment: for PCBs p = 0.356 and for PAHs p = 0.390. paper.

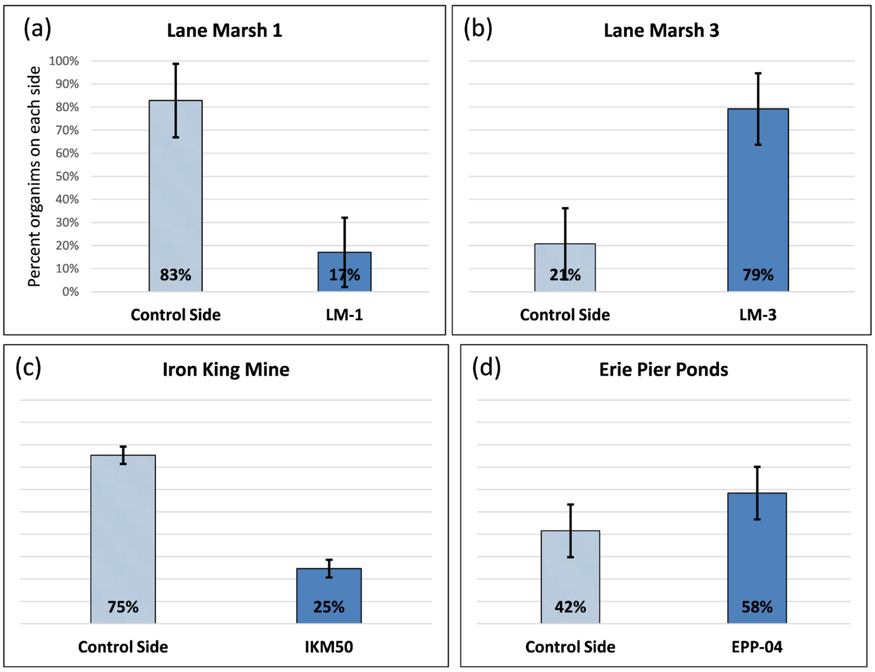

Figure 5. Results of sediment avoidance tests with *Hyalella* and sediment collected from four different sites: (a) (b) Bunker Hill Superfund site, (c) Iron King Mine Superfund site, (d) St. Louis River Great Lakes AOC site. Percentages found on control and test sediments sides after 24 hours. Error bars represent standard deviation of 4 replicate chambers.

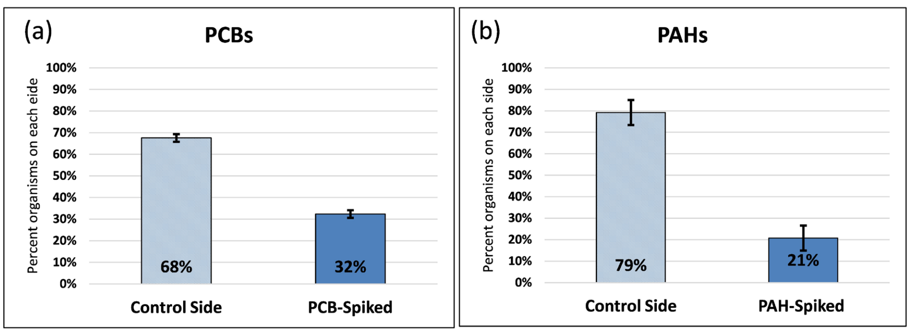

Figure 6. Number of organisms present on each side of chambers with half spiked with PCB or PAH mixtures at test end. Error bars represent standard deviations of 3 tests.

3.4. Testing for Other Behaviors

3.4.1. Phototaxis Results

One of the key test conditions was light. Should tests be run in continuous light, dark, or alternating periods of light and dark? For short tests, continuous light or dark made the most sense. The default continuous light, but some light vs dark tests were performed to examine if this is the best protocol, as there might be phototactic behaviors of the organisms that might affect testing. 24-hour tests were run with *Hyalella* and *Lumbriculus* in chambers modified by covering half of each chamber with black plastic (Figure 2(a)), with and without sediment in the chambers. *Hyalella* showed some signs of preference for dark when no sediment was present with 20% of organisms on the lighted side at test end, but clearly preferred light with sediment present in the chambers (Figure 7(a)), with 100% the organisms retrieved from the lighted side at test end. *Lumbriculus* showed no clear preference for light or dark (Figure 7(b)). Phototaxis tests were not done with *Chironomus*, as this organism had been dropped as a candidate by this time, due to mobility issues.

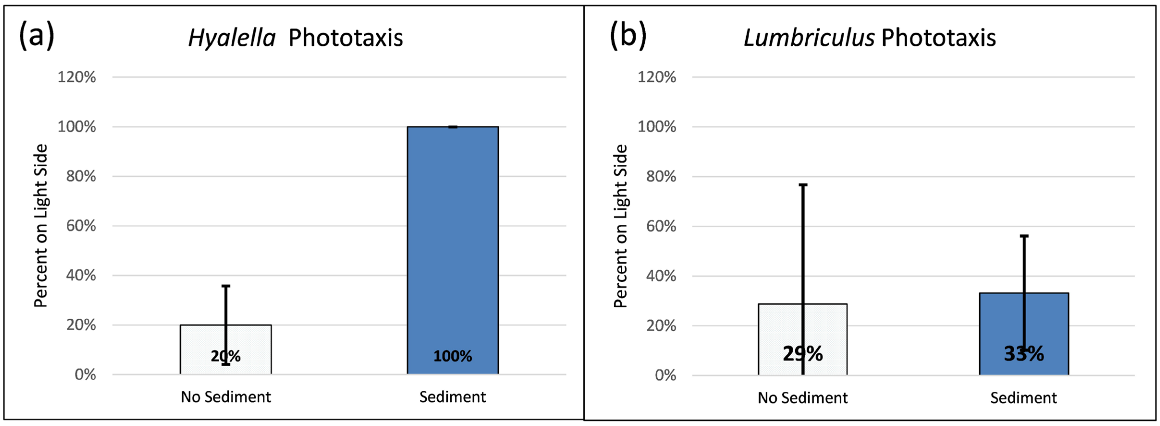

Figure 7. Phototaxis experiments (with sediment present). *Hyalella* showed a preference for dark conditions with sediment absent ($p = 0.0017$), a) but a very significant preference for lighted conditions with sediment present ($p < 0.00001$). Lumbriculus showed no preference for light or dark with sediment absent ($p = 0.257$) or b) present ($p = 0.079$). Error bars represent standard deviation in results from 4 replicate chambers.

3.4.2. Thigmotaxis Results

In the "transfer" dishes (Figure 8(a)) into which *Lumbriculus* from the main culture tank were placed (from which certain individuals were chosen and placed into test chambers), as well as in the test chambers themselves (Figure 8(b)), an "edge effect" or thigmotactic phenomenon was observed where individuals would move to the edges of the containers. Two "edge effect" experiments were performed, where plastic corers were used (diameter = 8 cm) to remove sediment from the center of the chambers (diameter = 13 cm), and the organisms found in this central core and in the remaining sediment around the edge were counted separately. Worms found on the surface were not counted in these analyses. In these tests, a higher percentage of worms were found around the edge than in the center (Figure 9), with p values of 0.0001 and 0.0003 for the two tests. A significant difference was also seen if number of worms is used in the analysis, with an average of 41 worm around the edge and 16 worms in the center found, and p values of 0.003 and 0.008 for the two tests. If viewed in terms of number of worms per unit area (cm^2), there was still a significant difference, with p values of 0.017 and 0.015 for the two tests. After going to the edges in the transfer dishes, worms could be seen forming tight clusters.

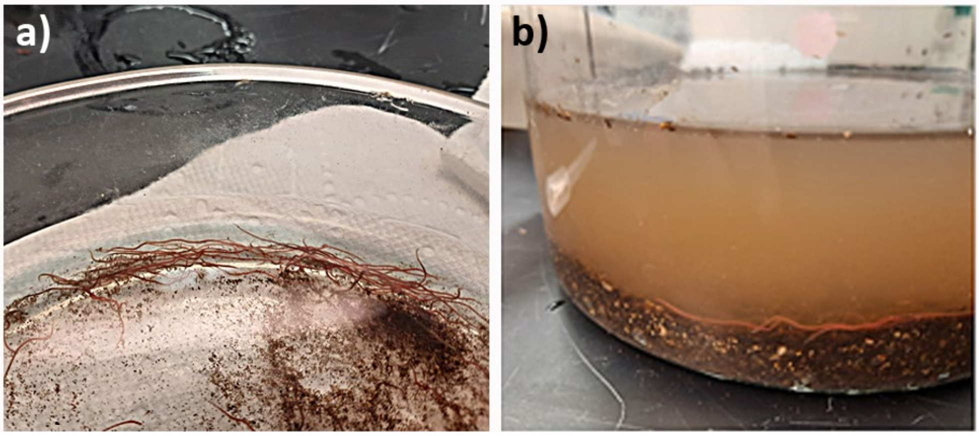

Figure 8. Thigmotaxis/Edge Effect in *Lumbriculus*. Whether in (a) containers with no sediment used in test setup, or (b) when put into test chambers at test start, the organisms would seek out edges/corners of the containers.

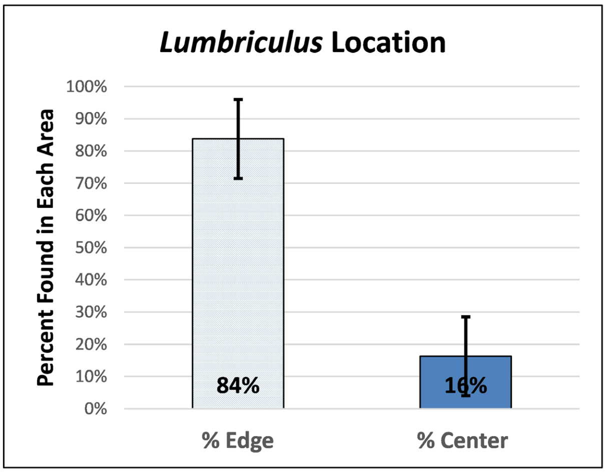

Figure 9. Results of the "edge effect" experiments, in which Lumbriculus were more likely to be found around the edges of the test chamber than in the center portion. Error bars represent the average standard deviation of two tests.

4. Discussion

Though invertebrates likely don't have a strict hierarchy of behaviors, a loose behavioral hierarchy concept based upon costs and benefits [33] of competing behaviors such as avoidance of predation, avoidance of toxic chemicals, foraging/feeding, mating etc., is likely a conceptual framework that can be useful in designing ecotoxicity tests and interpreting results. One way to conceptualize such a hierarchy would be a gradient of behaviors ranging from pure reactions (where response follows stimulus without any choice involved, similar to the knee-jerk reaction seen in humans) to actions (where an organism is actively choosing between multiple available behaviors). Though researchers are shining more light on the complexity of invertebrate behaviors and revealing that behaviors once thought of as pure reactions involve an element of choice [34], this kind of hierarchical framework can still have practical value. Strength, time frame, urgency, and consequences of an action could all potentially be factors in ranking. Under this conceptual framework, behaviors that are primarily reactions would be in the upper part of the hierarchy, in the sense that they would be expected to happen faster and stronger than actions that are less reaction, have larger elements of choice, and typically take place over a longer time frame (e.g., foraging, mating). Avoidance reactions, whether predator or toxic contaminant avoidance, have instant consequences regarding survival and reproduction. So, it makes sense that these would be strong reactions that would happen faster than say foraging behavior, and would likely be in the upper levels of such a hierarchy. Marklevitz *et al.* [35] saw a decrease in avoidance of harbor sediments with food added, associated with a longer exposure time (96 hours vs 24 - 72 hours). This could indicate that in the balance between the avoidance of contaminants and the attraction of food, the latter behavior has a longer time frame before it comes into play.

4.1. Test Conditions

4.1.1. Test Chambers

Of the 3 candidate test organisms, *Hyalella* was most able to quickly move through the test chambers. With their epibenthic lifestyle, they would feed and hide in the top layer of sediment and then swim through the overlying water to explore the chambers. As mentioned earlier, *Chironomus* were not as mobile and tended to quickly build cases on top of the sediment. *Lumbriculus*, moving through or on top of the sediment, could not move from point to point nearly as quickly as *Hyalella*. Though this mobility was an advantage in some respects, such as allowing a short test duration, there was a possible downside when using the same test chambers as the version used with the less mobile *Lumbriculus* and *Chironomus*. If disturbed by handling the test chambers at test end when trying to place the dividers at the midlines, these organisms could get from one side of the chamber to the other in the matter of a second. Organisms that had chosen to reside in the control sediment could end up in the test sediment or vice versa, affecting the results. So, it was decided that a version of chamber was needed for this organism, where the divider could quickly slide into place along the midline at test end. One caution in constructing this version of test chamber related to the observation that *Hyalella* have a habit of hiding (likely as a predator avoidance behavior), either in the top layer of sediment or squeezing into small spaces, if available. In an early version of this test chamber some *Hyalella* were found hiding in small gaps that in the caulking along the edges of the Plexiglas "runners" into which the divider goes at test end. So, smooth and gapless caulking of these runners is recommended.

4.1.2. Choice of Test Organism

An ideal outcome for a candidate organism in tests with only control sediment would be that there would be 10 organisms on each side of the chambers in all 4 replicate chambers, indicating that the organisms had spread themselves evenly within the chambers. Mobility tests and other tests with only control sediment examined how closely the candidate test organisms could achieve this, admittedly impossible, ideal behavioral criterion.

In the mobility tests, most *Chironomus dilutus* individuals stayed on the side of chambers where they were introduced. This was a key factor in dropping from the list of candidates for use in this sediment avoidance test (**Figure 4**, **Table 2**). Though *Lumbriculus variegatus* was initially considered a prime candidate, inconsistency in results of test with only control sediments by *Lumbriculus variegatus* was a key factor in not choosing this organism for this test. In two of the mobility experiments with *Lumbriculus*, the number of organisms was relatively equal on both the deployed and non-deployed sides, but in the other test, a clear majority ($p = 0.00000068$, **Table 2**) were on the deployed side at test end. A deeper dive into the mobility tests per chamber data (Sec. 3.1) showed that *Lumbriculus* was farther from this ideal of organisms spreading themselves out equally in the sediment inside the chambers than *Hyalella*. As many as 75% of chambers with *Lumbriculus* had a concentration of organisms on one side, as compared to 25% for *Hyalella*, Additional control experiments (Sec. 3.1) with *Lumbriculus* under alternate test conditions (e.g., 48-hour, dark) also showed this concentration of individuals on one side, adding to skepticism about the independent movement of *Lumbriculus variegatus.*

Sufficient sensitivity has been established for all 3 candidates due to decades of use in standardized tests [22] [23], and a wealth of studies in the literature that use these organisms. *Hyalella* may be the top candidate here, though, as only one of these 3 tests is for growth and survival [21], with *Hyalella* as the test organism.

Re environmental relevance, all 3 organisms are widely found in waterbodies in the environment, though *Chironomus* would have the edge here in terms of ubiquity. At Great Lakes AOC sites, amphipods of genera *Gammarus* and/or *Hyalella* are found in significant numbers at most sites (personal observation), while *Lumbriculus* are less frequently found. So, I would rank them *Chironomus* > *Hyalella* > *Lumbriculus* in terms of likelihood of being found at any site.

Re culturing, SOPs are readily available for all 3 organisms. Cultures of *Lumbriculus* are the easiest to maintain. A continuous culture can be kept in an aquarium for long periods of time by just supplying them with unbleached paper towels as food. *Chironomus* is the most difficult since, as the larvae mature, they go through metamorphosis and emerge as adults within a few months' time. Then adults must be mated and the culture restarted from eggs. *Hyalella* is of intermediate difficulty. So, I would rate them *Lumbriculus* > *Hyalella* > *Chironomus* in terms of ease of culturing. All 3 organisms are of equal ease of handing during testing.

Hyalella ranked first or second in each of these criteria, but especially its superiority in terms of mobility made it the clear choice for this test.

4.1.3. Test Duration

A 24-hour test was preferred in terms of observing a chemical avoidance reaction before other, possibly confounding, behaviors have a chance to interfere. In numerous tests with a variety of sediment types and contaminants, 24 hours proved long enough to observe and avoidance reaction from *Hyalella*. 48-hour tests were tried with *Lumbriculus*, but this did not solve the inconsistency issue. Other avoidance tests have been typically run for short durations. Earthworm soil avoidance tests are typically 48 hours [5] [6], though likely could be shortened. Frankenbach *et al.* [36] tested numerous contaminated soils and concluded that reducing the earthworm test duration from 48 to 24 hours did not result in a different assessment of the soils. Rastetter and Gerhardt [37] concluded that 16 hours was sufficient to run an earthworm avoidance test. The 24-hour duration of this sediment avoidance test may also be able to be shortened, and varying the duration would be a good area for future exploration.

4.1.4. Control Sediments

To produce control sediments the use of commercially available topsoils combined with a sieving process was explored, with the idea that, if successful, this would make the test more widely accessible. This attempt proved successful in that the test was robust enough to allow the use of variety of control sediments with varying properties (**Table 1**), including widely available and inexpensive commercial topsoils. The topsoils all had good levels of organic content, and though only 3 different site sediments were used as controls, they showed less variability than the site sediment controls (**Table 1**). The sieving process can also be used to make control and test sediments from different sources more consistent, in terms of particle sizes makeup. Having to sort through large debris at test end creates issues with recovery of test organisms. It increases more time to retrieve individuals and can reduce recovery percentage.

One hypothesis for *Hyalella* seeming to choose the Bunker Hill site test sediment LM-3 over the control sediment (Bearskin Lake) used in the first test with LM-3, involved the Bearskin Lake sediment having the lowest organic content (1.6%) of any of the control or test sediments (**Table 1**). So, perhaps the organisms were choosing the LM-3 sediment with higher organic content (20.0%) as a better food source. However, a subsequent test with LM-3 sediment and a different control sediment made from topsoil with higher organic content (9.8%) (**Table 1**), the results were essentially the same.

4.1.5. Other Test Conditions

Initial tests used moderately hard reconstituted water [27] that was specified by for use in most ecotoxicity tests at USEPA, Cincinnati. This requires a significant amount of effort and chemicals to produce. So, in line with our other efforts to make this test accessible, a transition was made early on to use distilled water, which caused no discernible issues.

There were no absolutely consistent trends regarding changes in the chemical and physical measurements (**Table 2**) of the overlying water between setup and takedown of tests, however conductivity typically increased and dissolved oxygen (DO) typically decreased. Paired t-tests showed changes ($p < 0.05$) in conductivity in 74% of tests and changes in DO in 84% of tests reported here. Of the test where there was a significant difference, 100% showed increases in conductivity, and 69% showed decreases in DO.

These measurements were meant to be used as a diagnostic tool if low recovery or confusing results were seen. In general, these test conditions were in the range seen in freshwater ecosystems. At test end, in only 2 of the 19 tests (**Table 2**) was the DO at or below the USEPA level of concern (2.5 mg/L) for ecotoxicity tests with *Hyalella* [21], *Lumbriculus* [22], and *Chironomus* [23]. The highest conductivity levels were seen in the test with IKM50 site sediment and in 2 of the mobility tests with *Lumbriculus* (**Table 2**), but recovery was good for all these tests; 95% in the IKM50 test, and 91% and 99% in the 2 tests with *Lumbriculus*. As opposed to the standard 10-day growth and survival tests [21], these are 24-hour tests, and the water chemistry values come from spot measurements. So, we know that, even when we see water chemistry levels that elicit some concern, the organisms have been exposed to them for <24 hours. Since the water chemistry would be expected to be the same on both sides of the chamber, readings outside of a desired range would not likely bias the organisms to choose one side over the other. Since *Hyalella* are sensitive to disturbance, renewal of the overlying water during the experiment does not appear to be a good option.

4.2. Mobility

As mentioned previously, the level of mobility was a key factor in choosing *Hyalella* for this test from our three candidate organisms. Though mobility may suffice as a general term to describe this criterion, the reality is more complex and involves interference of other behaviors such as case-building (*Chironomus*) (**Figure 10**) and possible clustering/aggregation and/or collective movement (*Lumbriculus*). An examination of the numbers and percentages of individuals on both sides of chambers at the end of the mobility test, as well as other control tests (Sec. 3.1) with varying test conditions (e.g., 48 hrs., dark) that were done to explore whether possible thigmotactic and/or aggregation behaviors in this organism were affecting independent movement, indicates that the *Lumbriculus* variegatus behavior is far from the ideal of organisms spreading themselves out evenly in chambers in control tests.

Our observations regarding the limited mobility of *Chironomus* agree with those of Baker and Ball [38] who found that the ability of *Chironomus tentans* larvae to move to find food appeared limited. Dornfield *et al.* [39] concluded that the non-avoidance of lethal levels of copper-contaminated sediments by *Chironomus riparius* larvae was likely due to lack of ability to move and that they became more sedentary after building cases, something that was observed in our mobility experiments. Wentsel *et al.* [40] found that *Chironomus tentans* avoided metals-contaminated sediments (Zn, Cr, Cd) at higher metal concentrations. One difference in the protocols between the 2 sets of experiments is that in our experiments, organisms were placed onto the contaminated sediments at test start, whereas

Wentsel *et al.* [40] placed them on the line where the contaminated test sediments and control sediments meet. In our experiments larvae were observed to start building cases soon after they arrived on the surface of the contaminated sediments. It's possible that they begin case building immediately when they sense they are surrounded by contaminated sediment, to provide a barrier between themselves and toxic sediment. However, if they are close enough to sense both contaminated and non-contaminated sediments, they may perceive a choice of 2 environments and may have a choice of 2 behavioral options to survive contaminated sediment; move a short distance or build a case.

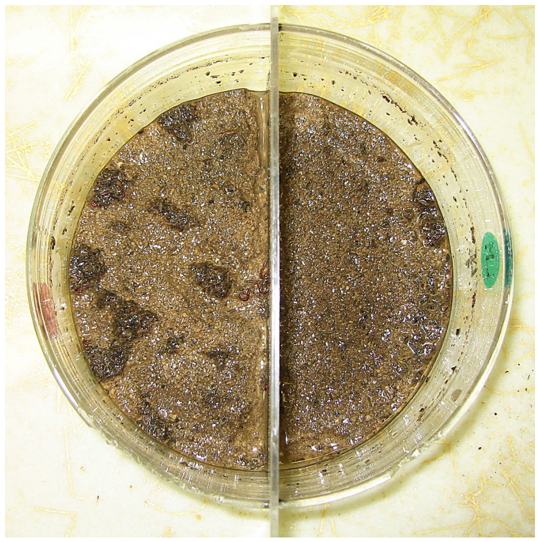

Figure 10. *Chironomus dilutus* cases on the surface of the sediment in a test chamber at the end of a 24-hour mobility test. Almost all the cases appear on the side of the chamber on which they were deployed at test start.

4.3. Site Sediments

In assessments of site sediments, results from avoidance tests correlated well with mortality and weight loss (**Figure 5**, **Table 3**). Avoidance of the metals-contaminated Iron King Mine sediment was seen where weight loss occurred but no significant mortality, giving evidence of the avoidance test being more sensitive.

As noted in the section on control sediments (4.1.4), more *Hyalella* ended up in the site sediment in two tests with LM-3 (the less-contaminated of the two stations from Lane Marsh) and two different control sediments (**Table 5**, **Figure 5**). It was concluded that as only one of the two control sediments had low organic content (Bearskin Lake sediment—1.6%) (**Table 1**), *Hyalella* were likely not choosing LM-3 as a better food source. The work of Suedel and Rodgers [41] and of Ankley *et al.* [42], which found that *Hyalella azteca* was tolerant of a wide range of organic content as well as particle sizes, also supports this conclusion. *Hyalella* survival in the Bearskin Lake sediment was 100% and 90% in the control sediment from topsoil, so there is confidence that the control sediments were non-toxic. So, the cause of *Hyalella* choosing the LM-3 sediment over control sediments appears not to be due to differences in organic content or toxicity between the control and site sediments. There were differences in particle makeup (sand, silt, clay) seen, as the control sediments had high levels of sand (41.7%, 54.1%) compared to the LM-3 sediment (0.0%) and the LM-3 sediment had high levels of silt (95.2%) (**Table 1**). As noted above, studies [41] [42] have shown *Hyalella* to be tolerant to a range of particle size makeup in terms of growth and survival. However, this doesn't tell us anything about whether these organisms may have different thigmotactic responses to particle size makeup, or how differences in these physical properties might affect choice of sediment as a refuge from predators, either of which could affect their choice of sediment in avoidance tests.

4.4. Spiked Sediments

The concentrations used in spiked sediments of total PCBs and PAHs, of approximately 50 and 107 mg/Kg respectively, are towards the high side of the range of concentrations found at contaminated Great Lakes AOC sites. For example, the highest measured total PCB concentration was almost 200 mg/Kg in pre-dredge sediment samples at the Ashtabula River AOC site [43]. Pre-remedy measurements of sediment contaminants at Ottawa River AOC site [44] found the highest values for total PCBs of 80 mg/Kg and for total PAHs of >300 mg/Kg dry weight. *Hyalella* avoided PCB and PAH mixtures at environmental levels at which there was little to no mortality in the accompanying acute tests, making this avoidance test more sensitive than an acute test to these persistent toxic chemicals which are targets of many sampling programs.

4.5. Other Behaviors

During these experiments, other behaviors were observed which have the potential to affect a test measuring avoidance of contaminated sediments; foraging, mating (pre-copulatory guarding), phototaxis, thigmotaxis, aggregation, predator avoidance. In addition to the convenience offered by a short-term ecotoxicity test, having a behavioral test that measures a "reaction" that happens in a short time frame lessens the chance of interference from these other behaviors. Attempts were made to account for behaviors other than chemical avoidance behavior, so that they would not interfere with the avoidance behavior and complicate interpretation of results. For instance, precopulatory guarding behavior is part of the mating behavior of *Hyalella* and other amphipods. This behavior involves mate pairing, with male amphipods grasping onto females for up to a week [45] [46]. Since large adult *Hyalella* were used in testing, and it was desirable that all test organisms act independently, use of paired *Hyalella* was avoided when setting up tests. A note was made of organisms found paired at test end, and fortunately these were rarely found. Allowing only a short period for mate pairing is perhaps another advantage of a short 24-hour test.

4.5.1. Phototaxis

Light vs dark experiments (Sec. 3.4, **Figure 7(a)**) revealed that *Hyalella* preferred dark over light conditions ($p = 0.0017$) with sediment absent from the chambers, but showed a very strong preference ($p < 0.00001$) for light with sediment present, as 100% of individuals were retrieved from the lighted side of the chambers. This provided further validation of the choice of continuous lighting during tests. A hypothesis for explaining the opposite phototactic responses of *Hyalella* depending on presence or absence of sediment is that this is another case of competing behaviors. Seeking a dark environment in absence of hiding places in sediment or associated debris may be predator avoidance behavior. However, in the presence of sediment in which to hide, light would likely aid in behaviors like foraging for food, mating, and predator avoidance. In experiments measuring percent of time that amphipods spent on the light and dark sides of chambers, Kohler *et al.* [47] concluded that both of their 2 test species were negatively phototactic. However, these tests were conducted in empty chambers with no sediment present. Though the numbers of *Lumbriculus* were not significantly different on the light or dark sides of chambers with sediment absent or present, the results of the experiment without sediment were affected by the clustering/aggregation or collective movement behavior observed in this organism. In that experiment, the numbers present on the light side of the 4 replicate chambers at test end were, 0, 0, 3, and 20. Without *Lumbriculus* aggregating in the one replicate chamber, it very well may have been concluded that the organisms preferred light in the absence of sediment. It is possible that they do prefer light in chambers without sediment, but that the aggregation behavior is obscuring their phototactic behavior. In any case, the behavior of the organisms under actual test conditions (with sediment present) is the more ecologically relevant, and more important regarding test protocols. *Lumbriculus variegatus* have been shown to have another light-related behavior, a response to moving shadow or sudden decrease in light intensity, presumably a predator avoidance behavior [48]. It is possible that this behavior, which involves retraction of the worms' tails protruding above the sediment to the air-water interface, could be triggered by opening of the incubator door during testing. However, this behavior has not been shown to include movement of the worms to new locations in the sediment, so it is unlikely that this behavior would lead to the local concentration or aggregation of worms seen in testing.

4.5.2. Thigmotaxis

Thigmotactic behavior is common among invertebrates, since it has value for organisms in reproduction and in defending against predation. Behavior that appeared to be thigmotactic was observed in both *Lumbriculus* and *Hyalella*. Experiments with *Lumbriculus* were done that showed more organisms around the edge after 24 hours than in the centers of chambers (**Figure 9**). These thigmotactic experiments were done to try to understand how the worms move and distribute themselves within the chambers and to address the inconsistency seen in avoidance tests with *Lumbriculus*. As *Lumbriculus* are known to often occupy the shallow margins of water bodies [48], it is possible that this edge effect thigmotaxis is useful in finding those margins. It is possible that touch-related thigmotaxis also plays a role in aggregation behavior.

There is evidence of thigmotactic behavior in amphipods. Kohler *et al.* [47] found positive thigmotactic behavior in a marine and freshwater amphipod (*Echinogammarus marinus* and *Gammarus pulex*). From observations over a period of many avoidance experiments, as well as observations of their interactions with sediment using a dissecting scope (where they were observed hiding under small bits of woody debris), *Hyalella* also showed evidence of thigmotactic behavior, seeking out edges and corners, nooks and crannies.

4.5.3. Aggregation

Though aggregation behavior may involve elements of thigmotaxis, we'll discuss it separately. There were concerns that aggregation may be the cause of the inconsistent results seen with *Lumbriculus*. Though, from these studies it can't be concluded that aggregation is the culprit, it is a strong suspect. The same kind of clumps or "clusters" that Zirbes *et al.* [49] observed in the terrestrial oligochaete, *Eisenia fetida*, were seen in the aquatic oligochaete *Lumbriculus* in the transfer dishes during test setup (**Figure 8(a)**). Ozkan-Aydina *et al.* [50] studied movement of tangled clusters of *Lumbriculus*, referring to them as "blobs". As additional evidence of possible aggregation affecting test results, in several tests conducted with control sediment on both sides of the chambers, most or all *Lumbriculus* individuals were found on one side of the chamber. In some tests, a large percentage of the worms were seen on the sediment surface. There could be opportunities for aggregation in these cases, and even a temporary aggregation could affect the distribution of organisms in the chambers. The Zirbes *et al.* [49] study indicated that there may be a threshold number of individuals needed before other worms begin to aggregate or join the group. Perhaps, in experiments where more worms move to the sediment surface there is an increased possibility of them achieving a threshold number and aggregation occurring. If the hypothesis of Zirbes *et al.* [49] that following chemical cues is an important mechanism for aggregation is correct, then a threshold level of worms may be needed to create a threshold level of attractant pheromone/chemical. Further shortening the test duration and/or decreasing the number of individuals per test could represent means for alleviating a threshold effect, if such effect exists. West and Ankley [18] did not report any inconsistencies in the results from their avoidance experiments with *Lumbriculus* or instances of aggregation. Though they do not report how many organisms they used per chamber, perhaps they used a smaller number of organisms per test, which prevented aggregation.

5. Conclusion

A sediment avoidance test has good potential to address the need for assessment of sediments at the large number of contaminated sites around the world. Of our three candidates for use in this test, *Chironomus dilutus* larvae, the amphipod *Hyalella azteca*, and the oligochaete *Lumbriculus variegatus*. *Hyalella* was chosen as the best candidate. *Hyalella* performed best in tests of mobility and independent movement within the test chambers. Sediment avoidance tests with *Hyalella* showed good sensitivity. *Hyalella* avoided both contaminated field-collected site sediments and sediments spiked with PCBs and PAHs at sub-acute concentrations. For avoidance and other behavioral ecotoxicology tests, as well as standard growth, survival, and reproduction tests, an awareness of other behaviors (e.g., phototaxis, thigmotaxis, mating) of the test organisms is recommended, as these behaviors can affect test results and their interpretation. Under the hypothesis that avoidance of chemical contaminants is more a reaction than a choice and happens on a shorter time scale than some other behaviors like foraging, an important advantage of avoidance tests is that they can be completed before these other more choice-based and longer-term behaviors have a chance to affect test results.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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