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Test Methods to Estimate the Acute and Chronic Toxicity and Bioaccumulation of Sediment-Associated Contaminants using the Aquatic Oligochaete, *Lumbriculus variegatus*

by

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OVERVIEW

There recently has been increased concern about the impacts of contaminated sediments on aquatic ecosystems. An important shortcoming of ongoing and planned sediment assessments, particularly from the standpoint of the U.S. Environmental Protection Agency (EPA), is the lack of standard techniques such as toxicity tests for evaluating potential impacts of sediment-associated contaminants. In this report we describe methods utilizing the aquatic oligochaete, Lumbriculus variegatus, to assess the acute and chronic toxicity, and the presence of bioaccumulatable compounds in contaminated sediments.

L. variegatus was chosen as a test species because: (1) it is ecologically relevant (i.e., it has a wide distribution and is an important component of aquatic food chains), (2) it is suitable for long-term testing and evaluation of chronic toxicity endpoints (e.g., growth, reproduction), (3) it is exposed via all important routes of concern, including ingestion of contaminated particles, and (4) it has sufficient biomass to assess bioaccumulation of contaminants. Also, this species is easily cultured under a variety of conditions (described herein) and is relatively easy to handle.

In addition to describing culture conditions, testing protocols (e.g., test lengths, sample sizes, feeding, etc.) and presenting the results of actual tests with the worms, we give specifications for an automated renewal exposure system which is suitable for testing contaminated sediments with a variety of species.

INTRODUCTION

Background - Recent surveys have amply demonstrated the extent of sediment contamination in the U.S. (U.S. EPA 1987). Sediment contamination problems at freshwater sites are prevalent throughout the country and include 41 major areas of concern in the Great Lakes alone (International Joint Commission 1985). To address issues associated with contaminated sediments, extensive remedial action planning, and in many cases Superfund activities, are underway at a number of sites. Major efforts such as the Assessment and Remediation of Contaminated Sediments program, through the EPA Great Lakes National Program Office, have been specifically mandated by law. Although there is a tremendous amount of activity focused upon contaminated sediments, standardized tools such as toxicity tests, for addressing existing or potential impacts of sediment-associated contaminants on aquatic ecosystems are not available. This shortcoming is of particular concern to the EPA, not only from an assessment standpoint, but because the lack of standardized test methods seriously impedes cohesive regulatory activities. The objective of this report is to present test methods, using a freshwater benthic invertebrate, for assessing the toxicity and bioaccumulation of sediment-associated contaminants.

Benthic Test Species - An important component of the assessment of contaminated sediments is the use of toxicity tests to evaluate impacts on benthic and upper-water column species. An "ideal" suite of toxicity tests would have a number of attributes including: (1) ecological relevance, (i.e., the organisms are potentially important species in the system(s) of concern), (2) ability to assess chronic endpoints such as reproductive effects, (3) protective of the most sensitive species in the system, (4) incorporate all possible routes of exposure (i.e., exposure to interstitial water, ingestion of contaminated particles), (5) ability to measure bioaccumulation to help assess possible impacts on higher organisms exposed through food chain transfer (biomagnification), and (6) utilize species amenable to culturing and

handling. Many different organisms and test endpoints have been proposed for assessing contaminated sediments (Giesy and Hoke 1990). However, the majority of these tests do not conform to all of the above criteria. For example, several studies have used standard upper-water column test organisms, such as cladocerans and fish, to assess the toxicity of contaminated sediments; however, these organisms generally are not relevant if species of concern are benthic, particularly in terms of adequately addressing all possible routes of exposure.

Three freshwater benthic organisms that have received significant attention with respect to testing contaminated sediments, and for which some test methods exist, are the amphipod *Hyalella azteca*, and the chironomids *Chironomus tentans* and *C. riparius* (Nelson et al. 1990). These species are ecologically-relevant, easily cultured and, because they are benthic and/or epibenthic organisms, are exposed to contaminants through various routes. Moreover, with these three species it is possible to examine a variety of endpoints, including those traditionally used as measures of chronic toxicity such as growth (*C. tentans*, *C. riparius*) and reproduction (*H. azteca*). However it is difficult to use these three species to evaluate the impact and/or presence of bioaccumulatable contaminants. Tissue masses obtained from *H. azteca* generally are too small to collect enough sample to analyze, and the life cycles of both chironomid species may be too rapid to adequately assess the presence of bioaccumulatable contaminants (particularly if tests are conducted with second instar larvae). Therefore, to compliment these three test species it would be useful to use an organism that could be used in long-term tests, and have sufficient biomass for analytical work. An excellent first choice would be the mayfly (*Hexagenia limbata)*, because it is considered to be relatively sensitive and has a large tissue mass. However, *H. limbata* currently cannot be cultured, so it is not a desirable species for routine testing. A logical alternative for long-term testing and associated analytical work involving bioaccumulatable compounds would be an oligochaete. Oligochaetes are ubiquitous in a variety of freshwater benthic habitats, they

have adequate body mass to perform analytical work with a logistically reasonable number of organisms, and certain species are easily cultured. Also, because of their prevalence in aquatic communities, oligochaetes are particularly well suited for use in bioaccumulation studies and in assessing potential for food chain transfer. Oligochaetes traditionally have been considered to be relatively tolerant of certain classes of contaminants. However, when assessing bioaccumulatable compounds, the tolerance of these species perhaps is a positive attribute (Schuytema et al. 1990).

Oligochaetes in Aquatic Toxicology - Various investigators have evaluated the use of distributions of oligochaete species as indicators of pollution in situ (Brinkhurst 1980; Spencer 1980; Lauritsen et al. 1985; Robbins et al. 1989). Results of these studies suggested that it was possible to identify oligochaete assemblages representative of the degree of organic contamination present at various study sites.

A number of researchers have used various oligochaete species in laboratory toxicity tests with pure compounds and/or contaminated sediments. Chapman et al. (1982a; 1982b) evaluated the toxicity of single chemicals, and combinations of chemicals, to up to 12 different freshwater and marine oligochaete species. They also investigated the influence of environmental variables, e.g., pH, salinity, temperature and anoxia, on the toxicity of the test chemicals to the oligochaetes. Wiederholm et al. (1987) described testing protocols for five species of freshwater oligochaetes, and noted that in sediments polluted by heavy metals, growth and reproduction were more sensitive endpoints than survival. Keilty et al. (1988a; 1988b) reported the toxicity of endrin to two species of freshwater oligochaetes, and concluded that a behavioral response (burrowing) of the organisms appeared to be a promising sublethal endpoint. Nebeker et al. (1989) investigated the toxicity of hexachlorobenzene to the freshwater oligochaete species, *Lumbriculus variegatus*, and found that tissue concentrations of the chlorinated compound as high as 24 μg/g had no adverse effects on survival, growth, or reproduction

of the worms. This agreed with observations made using "standard" test species, e.g., the fathead minnow, *Pimephales promelas*.

Oligochaete species have also been used to evaluate the bioaccumulation of nonpolar organic compounds in a laboratory setting. Oliver (1984) investigated the uptake of 24 chlorinated chemicals by a "natural" oligochaete assemblage exposed to Lake Ontario sediments for up to 110 d. He found that the uptake of chemicals from test sediments by the oligochaetes was approximately proportional to sediment concentrations of the chemicals. He also noted that the Kow (octanol water partition coefficient) of the test chemicals was integral in determining uptake kinetics. Connell et al. (1988) reanalyzed the data of Oliver (1984), and concluded that this phenomenon could be explained by the theory of equilibrium partitioning, assuming that the primary exposure phase was sediment interstitial water. Schuytema et al. (1990) exposed *L. variegatus* to hexachlorobenzene, and based on bioaccumulation, concluded that this oligochaete was a promising species for monitoring bioaccumulatable nonpolar organics in environmental samples.

Rationale for Species Selection - We chose the oligochaete, L. variegatus, as a test species for a number of reasons. *L. variegatus* is ecologically-relevant, it is prevalent throughout the U.S. and Europe, it is present in all five of the Great Lakes, and occurs in a great variety of sediment types (Chekanovskaya 1962; Cook 1969; Spencer 1980). It is possible to use the species to assess chronic toxicity endpoints such as growth and reproduction (Nebeker et al. 1989). *L. variegatus*, a truly benthic organism, is exposed to contaminants via all routes of concern, including ingestion of contaminated particles. Since a logistically reasonable number of organisms can provide adequate tissue mass for residue analyses, (i.e., about 80-100 per g wet weight) this species is ideal for long-term bioaccumulation studies. *L. variegatus* is extremely easy to culture and maintain in the laboratory year round, and is relatively easy to handle (e.g., removal from test sediments).

Finally, there is some information in the open literature describing the use of *L. variegatus* in laboratory toxicity tests (Nebeker et al. 1989; Schuytema et al. 1990).

METHODS AND MATERIALS

General

Life History and Life Cycle - L. variegatus normally dwells in silty and sandy sediments at depths of 2 to 60 meters in reservoirs, rivers, lakes, ponds, and marshes (Chekanovskaya 1962). The anterior half of an individual is usually buried in the sediment while the posterior half undulates in the overlying water to achieve respiratory exchange. While feeding on organic material in the sediment the worms will tunnel to a depth corresponding to the aerobic zone of the sediment, and do not penetrate the anaerobic zone appreciably.

L. variegatus has a sexual reproductive potential. However, little is known about it, and Chekanovskaya (1962) states that individuals with sexual organs are extremely rare. The worm's most common mode of reproduction is architomy, where new individuals are budded off the anterior end of the parent, and subsequently are replaced with eight new segments. Body length varies from 40-90 mm and diameter varies from 1.0 to 1.5 mm. Newly hatched worms have never been observed in our cultures, which consist solely of adults of various sizes. In our laboratory, the population doubling rate is approximately 10 to 14-d at 20°C. The population growth rate seems to be related to how quickly the neonates grow to a size where they will again divide.

Collection - L. variegatus may be found in many field locations. While it is possible to start a culture with these organisms, the easiest way is to obtain them from another laboratory. This eliminates the need to find an adequate number of worms and positively identify the brood stock, which can be difficult because of the morphic structures necessary for identification (Pennak 1978). Brood stock can be successfully shipped by placing 100-200

organisms in 200 mL of clean water and protecting them from being crushed or exposed to extreme temperatures. Since these organisms are quite tolerant to changes in temperature, dissolved oxygen (DO) and pH, elaborate acclimation schemes generally are not necessary before they are introduced to new culture systems. Several laboratories which currently maintain *L. variegatus* cultures, as well as the appropriate contacts, are listed in Table 1.

Culturing - L. variegatus have been successfully cultured year round in our laboratory using a variety of substrates and foods. The substrates used include: shredded brown paper toweling (our recommended choice because of ready availability, relatively well defined characteristics, and ease of harvesting the worms), presoaked, dried maple and/or poplar leaves, or organically rich, clean sediments. The substrates are placed on the bottom of an aquarium at 3 to 6 cm depth. Filtered Lake Superior water flows through 57 L aquaria (100 mL/min) supplying bacteria, fungi, algae and other organisms, all of which contribute to the culture/substrate food complex. This substrate is supplemented twice weekly with about 3 mL of settled, hatched brine shrimp nauplii, and 1 to 2 g of U.S. Fish and Wildlife Service-certified salmon starter every 1 to 2 wk depending upon biomass in the aquarium. We have had excellent success in culturing the worms with about 50 snails, Helisoma sp., which process the substrates reducing them to particle sizes that the worms can easily ingest. The worms can be cultured without snails, but they seem to reproduce more efficiently in their presence. Culture tanks do not require frequent cleaning when the snails are present.

Cultures must not be allowed to become anaerobic; this possibility is minimized by culturing the worms in a flow-through system. However, with proper aeration and food limitations it is possible to culture L. variegatus in a static system. A rule-of-thumb is, if the culture shows signs of degraded water quality (e.g., smells offensive, has low DO, etc.) it has been over-fed. This can be corrected by increasing the aeration rate and/or water exchange rate. The optimal water flow in any particular system will be

related to biomass and biological oxygen demand, and therefore needs to be monitored and adjusted as appropriate. We recommend that DO in the cultures be maintained at $\geq$ 60% saturation.

L. variegatus production increases with increasing temperature, but the organisms should not be cultured above 25°C. Ideally they should either be cultured within 2°C of the projected test temperature, or be temperature-acclimated for at least 24-h before testing.

Light is supplied by a single cool white 20 watt fluorescent bulb for 3 aquaria, situated about 60 cm above the tank bottoms. The photoperiod is 16 h light: 8 h dark throughout the year.

Handling - If paper toweling is used as the substrate, the worms and snails will process it to a wet powder in 1 to 2 mo. The worms inhabiting this wet powder are easily collected for testing. They can be removed from the culture aquaria with a dipnet and placed in a flat, shallow-sided pan, rinsed, counted and distributed. A pipette made from 4 mm I.D. glass, 20 cm long and fire polished on both ends, fitted with a pipette bulb, is used for handling. The worms will clump and can be separated by a stream of water from the pipette. It is not advisable to separate the worms mechanically since they can be easily injured. Damaged or inactive worms should be discarded.

Testing

Test System - Toxicity and bioaccumulation tests are conducted in a system of 12 exposure tanks, 30 cm x 16 cm x 13 cm high (Fig. 1). This system is an adaptation of the serial diluter described by Benoit et al. (1982). The tanks have self-starting standpipe siphons 9.5 cm high on one end (Figure 1), which automatically vary the height of the water in the tank by about 2.5 cm. The tanks each hold eight 300 mL high form glass, berzelius exposure beakers. Each beaker has two 2 cm holes drilled at a 180° angle from one another other and 8 cm from the bottom of the beaker (Fig. 1). The holes are covered with 60 mesh stainless steel screen held in place with silicone adhesive. When a

test is initiated, 100 mL of sediment is placed in the bottom of the beaker, the beaker is then placed into the test tanks, leaving approximately 100-150 mL of overlying water in the beaker. Each different sediment in a test has its own test tank to prevent cross contamination with other sediments. As the water rises and falls in the tank (effected by the self starting siphons), the water in each test beaker is exchanged equally with tank water. With 12 tanks in a diluter system it is possible to test 12 different sediments with multiple replicates and one species, or several species simultaneously. For example, we have simultaneously tested L. variegatus, H. azteca, and C. tentans larvae in this system.

A timer-controlled solenoid valve on the water supply line of the diluter enables us to control the water replacement times in the test tanks. This allows adjustment of water flow through the test tanks such that adequate DO, pH, ammonia and test temperatures can be maintained without excessively stripping the test sediments of the inherent toxic materials. This system could be best described as an automatic renewal system, with a range of renewal from static to 10 tank replacements per 24 h.

This basic test system has also been used with L. variegatus for bioaccumulation studies with nonpolar organic chemicals (e.g., pesticides, PCBs). In these studies, a larger volume of sediment is required to expose enough organisms for analytical work. To accomplish this, the exposure beakers are not used and varying amounts of sediment are added directly to the test tank.

Before testing it is important to remove any toxic residues remaining in the system. A good routine to clean residues from the test system is: (1) soap and water wash, (2) dry, (3) rinse with acetone, (4) dry, (5) acid soak for 30 minutes in 10% nitric acid, (6) rinse in distilled water and (7) dry. The system then should be flushed with clean water for at least 4 d before initiation of further tests. If it is known that there are no organic contaminants the acetone rinse may be omitted.

Although the exposure system described above is convenient for evaluating toxicity and/or bioaccumulation of sediment-associated contaminants, a variety of other types of systems can be used for testing L. variegatus. For example, it is possible to test the organisms in beakers containing sediment, with overlying water that is renewed often enough to maintain adequate water quality. Alternatively, we have used L. variegatus in water-only exposures with various chemicals and aqueous sediment test fractions (e.g., sediment interstitial water), also in a renewal situation. We feel that L. variegatus can be tested in a variety of systems and situations, providing that appropriate controls are maintained.

Toxicity Tests - Whole sediments are homogenized so that all test beakers receive uniform 100 ml samples. The beakers are then placed into the appropriate test tanks where the water is flowed slowly into the beakers. The system is operated for 24 h to equilibrate. A renewal rate of two to six water turnovers daily is maintained. Organisms (usually 10) are randomly placed into the beakers and the time noted as the start of the test. We have run toxicity tests ranging from 4 d to 28 d; however, we generally do not recommend the use of tests of less than 10 d in duration. At the end of the test, worms are removed by sieving with a standard test sieve, (No. 35, 500 μm) counted, placed in dried, pre-weighed aluminum weigh boats, a drop of ethanol added (to immobilize the worms), and the weigh boats placed into a drying oven at 100°C for 4 h to 24 h, after which the worms are weighed. Individual dry weight ranges from 0.5 to 2.2 mg per organism. The toxicity test endpoints include survival, reproduction, and growth, expressed either as total biomass or biomass per individual. Note that in tests greater than 10-d in length, L. variegatus usually will reproduce, and given its mode of reproduction (i.e. architomy), it is impossible to differentiate between young and adult organisms. This necessitates the treatment of survival and reproduction as a single endpoint.

In some instances test sediments will contain oligochaetes which might be confused with L. variegatus; however, in our experience we have not noted that other oligochaete species present in test samples resembled L. variegatus to the extent that problems were encountered (e.g., it is quite easy to differentiate between L. variegatus and the ubiquitous tubificid species). If problems are encountered where oligochaetes present in test sediments resemble L. variegatus, more extensive morphological analysis may be required to interpret test data. There is the possibility that L. variegatus may actually be present in the test sample. In that case, the sediment probably is not toxic to the species. Some have removed "extra" species in test sediments by sieving, drying or freezing samples prior to testing; however, we strongly advise against this because of the potential for dramatic changes in the chemical and toxicological nature of the sediments (e.g., oxidation of sulfide with the concomitant release of metals; Ankley et al. 1990; DiToro et al. 1990). To evaluate whether oligochaetes are present in test sediments, we recommend routinely running an extra beaker with no organisms added, which then is seived and checked for native oligochaetes when the exposure is completed. This type of a control will thus alert the investigator to the possibility of ambiguous test results.

Bioaccumulation Tests - Bioaccumulation tests generally are set up with larger volumes of sediment because a larger biomass of worms is needed (particularly in the case of nonpolar organics). Sediments are placed in the test tank at 1.5 to 2.0 L per tank. The sediments are allowed to settle for 24 h before the test begins. The worms are weighed and placed in the sediment at the mass required for analytical measurements. We use 1.0 g wet weight of worms (about 80-100 organisms) per tank. At this stocking rate, if it is assumed for example, that 1 g of worms is exposed to 2 L of sediment, with an organic carbon content of 5% (dry weight, assuming 50% water), this would yield a sediment carbon/organism carbon of about 50 to 100, which should be high enough to ensure that the worms do not exhaust the bioaccumulatable nonpolar

organic compounds in the sediments. As for toxicity tests, the system is renewed with two to six water volume turnovers daily throughout the test. The test is conducted for the desired length of time (we have run bioaccumulation tests for up to 60-d), the worms are removed from the sediment with the standard testing sieve (No. 35, 500 μm) and put in clean water, where they are held for 24 h to purge gut contents, and then rinsed again to remove remaining debris. The worms are placed into acid- and solvent-cleaned glassware prior to chemical analysis.

Feeding - We have maintained L. variegatus in Lake Superior water without feeding for 35 d, with no loss of animals. Because feeding alters the organic carbon content and possible bioavailability of contaminants in sediments, feeding the worms during bioaccumulation tests is not recommended. However, it is necessary to feed the worms a minimal amount of food when testing for toxicity, because if one of the test sediments is low in organic carbon, it may appear to be toxic, when, in fact, adverse impacts (i.e., low reproduction and/or weight gain) may be caused by a lack of nutrients.

A series of tests was conducted to help define the influence of organic carbon on L. variegatus growth and reproduction. A clean sediment with low organic carbon (1 to 2% organic carbon) was compared to a clean sediment with high organic carbon (8 to 10% organic carbon). In initial experiments, sediments were tested for 12 days with variable worm loading rates (2, 5, 10 or 20 organisms per 100 mL sediment), using four replicate beakers per treatment. Reproduction and biomass were consistently lowest at all stocking rates in the low organic carbon content sediment. For example, reproduction in the high organic carbon sediment was 62, 25, 63 and 72 greater than in the low carbon sediment for the 2, 5, 10 and 20 worm stocking rates, respectively.

A second test was conducted, using various feeding rates, where L. variegatus reproduction and growth in the sediment with low organic carbon, as well as in sand with no organic carbon, were evaluated. Initial worm loading was 10 animals per 100 mL of sediment. Feeding rates were 0, 0.11, 0.33, 1.0

and 3.0 g of U.S. Fish and Wildlife Service-certified salmon starter stirred into each 100 mL of sediment or sand at the beginning of the test. Water was renewed at two volumes per day. Water quality was significantly impacted by all treatments receiving more than 0.11 g of salmon starter, and most worms died. However, animal numbers and biomass at the 0.11 g feeding level in both the low organic carbon sediment and the sand were comparable to values obtained using the high organic carbon sediment described above.

A third test was conducted in which triplicate treatments were fed 0, 20, 40 or 80 mg of salmon starter settled to the sediment surface every third day, rather than the larger, initial amount stirred into the sediment, as in the second test. The same low organic carbon sediment and sand as in the second test were used. Table 2 gives the mean numbers of worms, total biomass, and individual weights of worms for each treatment. Numbers of worms, total biomass and individual weights were higher in the 20 mg/feeding than in the treatments that were not fed. The number of worms, total biomass and mean weight/worm were reasonably similar in the 20 mg/feeding for both the low organic carbon sediment and sand. However, the biomass was slightly greater in the sediment than in the sand.

When the feeding rate was increased from 20 mg/feeding to 40 mg/feeding the mean number of worms decreased from 25.7 to 18.3 in the low carbon sediment but increased from 23.3 to 25.0 in the sand. Although total biomass in the low organic carbon sediment and sand was similar, mean weight per worm was elevated in the low organic carbon sediment. The 80 mg/feeding treatment showed an increase in individual worm weights but a decrease in worm numbers and biomass, therefore, this feeding rate was definitely detrimental to the worms. The choice between the 20 mg and 40 mg feeding rate is more difficult to make. However, the 20 mg/feeding treatment provided fairly good agreement between the three measured parameters in the two sediment types, while the 40 mg/treatment differed in worm numbers and mean weight/worm between the two sediments, possibly indicating over-feeding.

Based on results from this test, we recommend the addition of 20 mg of trout starter per 100 mL sediment on every third day of a test. The food should be added to the water overlying the sediment, stirred and allowed to settle to the sediment surface. Our experience in feeding L. variegatus during testing is somewhat limited; for example, there may be foods better than salmon starter to use when testing and we are investigating this.

Because of varying biological oxygen demands for different sediments, and the potential for exacerbation of biological oxygen demand by feeding, DO must be closely monitored to assure that 60% of saturation is maintained. If DO falls below 60% of saturation, the water flow rate must be increased or static tests must be aerated; it also may be necessary to decrease the feeding rate.

Control Sediments - The issue of control samples in toxicological research with sediments is extremely complex; to date, there have been no entirely satisfactory methods developed to address the issue of a suitable control for sediment toxicity tests. A "true" control would exactly mimic the test sample in all respects except for contaminants; this is a nearly impossible challenge. The basic problem in identifying suitable controls is related to the fact that physical/chemical conditions, such as particle size or organic carbon content, in the test sediments can markedly influence responses of test organisms. Therefore, less than optimal physical/chemical characteristics in the test sediments could result in adverse effects in the toxicity test, which may be independent of the presence of toxic contaminants. Probably the best treatment of the issue of appropriate control sediments was a statistical technique presented by DeWitt et al. (1988); however, this approach requires far more data than currently are available for L. variegatus.

The inability to define "true" controls for the L. variegatus test may not be a serious problem. Based upon work by other researchers (e.g., DeWitt et al. 1988), it appears that two of the most important physical/chemical characteristics which influence the health of benthic species in clean

sediments are (or are related to) particle size and organic carbon. In the tests we have performed, it appears that L. variegatus is relatively tolerant of a wide range of particle sizes. For example, we have tested the worm successfully in sand. Of course, some common sense must be used, e.g., it would be inappropriate to test the worm in a substrate consisting solely of rocks. With respect to organic carbon content, as described above, L. variegatus growth and reproduction can be quite dependent upon this variable. However, the incorporation of routine feeding should help negate the effects of organic carbon on the results of the worm test.

Although it is not now possible to define controls for sediment toxicity tests in a traditional sense, the researcher must have something to compare test results to in order to determine whether adverse effects have occurred. Two options are available. The first approach is to generate baseline data for statistical comparisons through a biological control, i.e., a clean sediment in which the organism is known to live, grow and reproduce in an acceptable manner. This type of control serves as a measure of test organism health and therefore, serves as a basis with which to compare results generated using test sediments. Typically, a biological control will be a sediment routinely used by and readily available to the testing laboratory. For example, our biological control for many studies is a sediment from West Bearskin Lake in northern Minnesota. A second option for generating data for statistical comparisons features the use of an ostensibly clean reference site (or sites) from the study location (or a nearby system). Results of toxicity tests with the test sediment(s) can then be compared statistically to results generated using the reference samples. In all instances, a biological control should be run simultaneously with the test sediment. A reference site also may or may not be run, depending upon study objectives.

Sample Sizes and Statistical Techniques - The goal of any toxicity test, must, in large part, dictate the number of replicates utilized for testing. For example, in some instances it may be necessary to

be able to identify statistically a 20% change from control values, while in other cases the ability to discern a 100% difference is adequate. Rather than attempting to make recommendations concerning sample sizes to be used, we evaluated several of our data sets to determine approximate numbers of replicates required to detect specific between sample differences in the biological endpoints. The technique used for this exercise is described by Steel and Torrie (1980; pg. 117); among the assumptions made for these calculations was that our data were normally-distributed. The assumption of data normality was evaluated and found to be reasonable using the NSCORES function available through the Minitab^{R} statistics package (Ryan et al. 1980). Sample sizes were calculated assuming a two-tailed alternative with the probability of Type I error (α) of 0.05 and Type II error (β) of 0.20. Variance estimates for the calculations for the various biological endpoints in the L. variegatus test were derived from a series of 10-d exposures conducted with multiple replicates of sediments from sites exhibiting a wide range of toxicities. Except in instances where all the worms were killed (i.e., no variance), variance across the sites was reasonably similar. Table 3 presents results of the sample size calculations for the 10-d exposures. In our experience thus far, results of longer-term tests with the worm (e.g., 28 d) are less variable than those from short-term tests (e.g., 10 d), so these sample size estimates should be useful for tests of 10 d or longer. When using these sample size estimates, notice that they were derived based on variance observed under our test conditions, and may not apply to all tests in which L. variegatus is used.

Results of toxicity tests with L. variegatus can be analyzed statistically using a variety of standard techniques. We evaluate nontransformed toxicity data using analysis of variance followed by a multiple comparison technique, such as Dunnett's test. Recently, Hoke et al. (1990) described the use of analysis of variance followed by linear orthogonal contrasts to evaluate the results of sediment toxicity tests; this approach may be more appropriate for assessing "groups" of samples than for the more

commonly utilized multiple comparison techniques. In instances where only one sample is being compared to a control or reference, standard t-tests also would be suitable for data analysis. The reader is urged to consult a standard biometrics text (e.g., Steel and Torrie 1980) for further guidance concerning possible statistical tests.

Quality Assurance

Quality assurance procedures associated with good laboratory practices were followed throughout this research. Because quality assurance is an essential component of developing a new methodology, descriptions of these procedures are included throughout the text.

RESULTS AND DISCUSSION

Presented below are two case studies in which L. variegatus was used to: (1) assess the bioaccumulation of metals (cadmium, nickel) from sediments from the Foundry Cove Superfund site in New York, and (2) assess the toxicity of sediment samples collected from the copper-contaminated Keweenaw Waterway/Torch Lake system in Michigan. For both studies, unless otherwise noted, basic test conditions were those described in Materials and Methods.

Case Study One - Foundry Cove - Recent studies at Duluth and the EPA laboratory at Narragansett have revealed that it is possible to predict the bioavailability and toxicity of cationic metals in sediments through determination of acid volatile sulfide (AVS) content (Ankley et al. 1990; Carlson et al. 1990; DiToro et al. 1990). Briefly, sulfides in sediments, normally precipitated as iron monosulfides, will preferentially bind to a number of metals of environmental concern including cadmium, nickel, lead, copper, and zinc, thereby reducing their bioavailability. These reactions proceed on a unimolar basis, i.e., one mole of metal will react with one mole of sulfide. Thus, as long as the molar ratio of metal/AVS is less than one,

metals in sediments are not bioavailable; however, when the ratio exceeds one, metals theoretically become bioavailable.

Initial studies which focused upon validating the metal/AVS hypothesis used toxicity as an endpoint, i.e., determination of mortality of relatively sensitive species, such as amphipods, in sediments with varying metal/AVS ratios (DiToro et al. 1990). Bioaccumulation is another possible endpoint to assess bioavailability of metals in sediments. Therefore, we designed a series of studies with L. variegatus to determine whether metal/AVS relationships in test sediments also could be used to predicted bioaccumulation of metals by benthic species.

Test sediments used for these studies were from Foundry Cove, New York, a Superfund site contaminated with both cadmium and nickel by a battery plant. Detailed experimental protocols are given by Ankley et al. (1990). Briefly, duplicate beakers with 20 L. variegatus were exposed to Foundry Cove sediments from 17 sites for 10-d, after which the worms were depurated for 24 h in clean Lake Superior water and then analyzed for metals. Figure 2 summarizes data from these experiments. In the top panel (Fig. 2a), concentrations of metals in L. variegatus (expressed as the molar sum of cadmium plus nickel) are plotted against total metal concentrations in the test sediments; it is apparent that the total metal concentration in the sediments was a poor predictor of metal concentrations in the worms. In the lower panel (Fig. 2b), concentrations of metals in L. variegatus are expressed relative to molar metal/AVS ratios in the Foundry Cove sediments; overall, when metal/AVS ratios were less than one, metal concentrations in the worms were uniformly low; however, when the metal/AVS ratio exceeded unity, elevated tissue concentrations of metals were observed. Thus, the metal bioaccumulation data derived from the L. variegatus exposures provided additional support for the critical role of AVS in determining metal bioavailability in sediments.

Case Study Two - Keweenaw Waterway/Torch Lake - As part of another ongoing study at Duluth, a series of sediment samples were collected from the Keweenaw

Waterway/Torch Lake system in the Keweenaw Peninsula of Michigan. Torch Lake also is a Superfund site which has been contaminated with significant amounts of copper associated with mine tailings. Sediments from the system reportedly have low benthic diversity and are toxic to some upper-water column test species (e.g., Daphnia magna) (Kraft and Sypniewski 1981; Malueg et al. 1984). One of our objectives, therefore, was to determine whether sediments from the system also were toxic to benthic species such as L. variegatus.

Exposures were conducted with three sediment samples from the system, one from Torch Lake, and two from the Keweenaw Waterway; the biological control for the test was sediment from West Bearskin Lake. Exposures were conducted using 10 organisms per beaker with eight replicates per site (we were interested in detecting relatively small differences in toxicity). Sediments from all three test sites significantly decreased survival/reproduction of the organisms in 10-d exposures (Table 3). (Note the use of survival/reproduction as a single variable). Total biomass also was significantly reduced in the Keweenaw/Torch Lake sediments relative to the West Bearskin control; however, mean weight per worm was not significantly decreased. Although in this particular test, survival/reproduction (and subsequently total biomass) were significantly affected, while growth (weight per organism) was not, with other samples we have seen the opposite effect, i.e., growth, but not survival/reproduction was reduced. Overall, our test results from the Keweenaw/Torch Lake sediments study indicate that sediment toxicity may be an important factor contributing to low benthic diversity in the system.

SUMMARY AND CONCLUSIONS

Methods are presented which detail the use of the aquatic oligochaete, L. variegatus, in toxicity and bioaccumulation tests with contaminated sediments. Although attention must be given to various issues in the broad area of sediment testing (e.g., identification of suitable controls) as well as specific factors in the L. variegatus test (e.g., feeding), we feel that,

based on our experience with this species over the past 2 years, it is an excellent organism for sediment assessment work. It offers great flexibility with respect to toxicity endpoints as well as test lengths, and is suitable for assessing bioaccumulative chemicals. Moreover, L. variegatus is readily cultured under a variety of conditions and is amenable to handling.

Although test methods presented in this report for L. variegatus are as far advanced as for any freshwater benthic species (e.g., H. azteca, C. tentans, C. riparius; Nelson et al. 1990) it should not be construed that this test has been field-validated. In order to provide regulators with a legally-defensible set of tests, it is imperative that methods be validated by comparing results obtained in the tests to benthic communities in situ. This is the only way to ensure that the test methods are indeed protective of benthic species. ERL-Duluth currently is initiating field validation studies for L. variegatus, H. azteca, and C. tentans toxicity tests.

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Table 1. Some laboratories currently maintaining cultures of *Lumbriculus variegatus*.

Laboratory	Contact Person	Phone
Environmental Research Laboratory-Duluth 6201 Congdon Boulevard Duluth, MN 55804	Gary Phipps	218-720-5571
Environmental Research Laboratory-Corvallis 200 S.W. 35th Street Corvallis, OR 97333	Alan Nebeker	503-420-4875
Center for Lake Superior Environmental Studies - University of Wisconsin-Superior Superior, WI	Larry Brooke	715-394-8318
Environmental Sciences Section Health and Environment Laboratories Eastman Kodak Company Building 306 Rochester, NY 14650	William Ewell	716-588-4528

Table 2. Reproduction, biomass, and individual organism weights in a low organic carbon sediment and sand with 4 different feeding rates. All values are expressed as the mean ± standard deviation for 3 replicates. Each replicate started with 10 animals.

Feeding Rate	Number of Worms		Total Biomass (mg)		Biomass/Worm (mg)	
	Sediment	Sand	Sediment	Sand	Sediment	Sand
0 mg	16.7 ± 0.58	23.0 ± 2.6	13.94 ± 0.50	13.41 ± 1.04	0.84 ± 0.05	0.59 ± 0.05
20 mg	25.7 ± 2.5	23.3 ± 2.5	24.74 ± 0.91	19.41 ± 1.91	0.97 ± 0.06	0.83 ± 0.04
40 mg	18.3 ± 4.9	25.0 ± 5.0	24.90 ± 2.48	21.92 ± 0.73	1.40 ± 0.02	0.90 ± 0.16
80 mg	10.7 ± 2.1	15.3 ± 2.5	15.35 ± 1.05	15.24 ± 1.53	1.46 ± 0.02	1.00 ± 0.06

Table 3. Approximate sample sizes (n) for detecting among sample differences in biological endpoints (δ) for Lumbriculus variegatus toxicity tests. Sample size is expressed as number of beakers (with 10 worms/beaker).

Endpoint	δ	n
Survival/Reproduction	1	198
(number of organisms)	2	50
	3	22
	4	13
	5	8
	6	6
	7	4
	8	3
	9	3
	10	2
Biomass	1	126
(mg/beaker)	2	63
	3	14
	4	8
	5	5
	6	4
	7	3
	8	2
Biomass	0.1	71
(mg/worm)	0.2	18
	0.3	8
	0.4	5
	0.5	3
	0.6	2

Table 4. Toxicity of test sediments from Torch Lake (TL) and the Keweenaw Waterway (KW1, KW2) relative to the West Bearskin Lake (WBL) control. Data are expressed as the mean ± standard deviation for eight replicate determinations.

Site	Survival/Reproduction	Total Biomass (mg)	Biomass/Worm (mg)
WBL	14.40 ± 1.92	14.67 ± 1.41	1.03 ± 0.07
TL	7.13 ± 0.99*	5.02 ± 1.92*	0.72 ± 0.30
KW1	6.90 ± 4.20*	5.08 ± 3.51*	0.83 ± 0.20
KW2	9.13 ± 0.64*	8.65 ± 1.12*	0.95 ± 0.10

* Differed significantly ($p < 0.05$) from the WBL control.

Figure 1. A testing system for contaminated sediments.

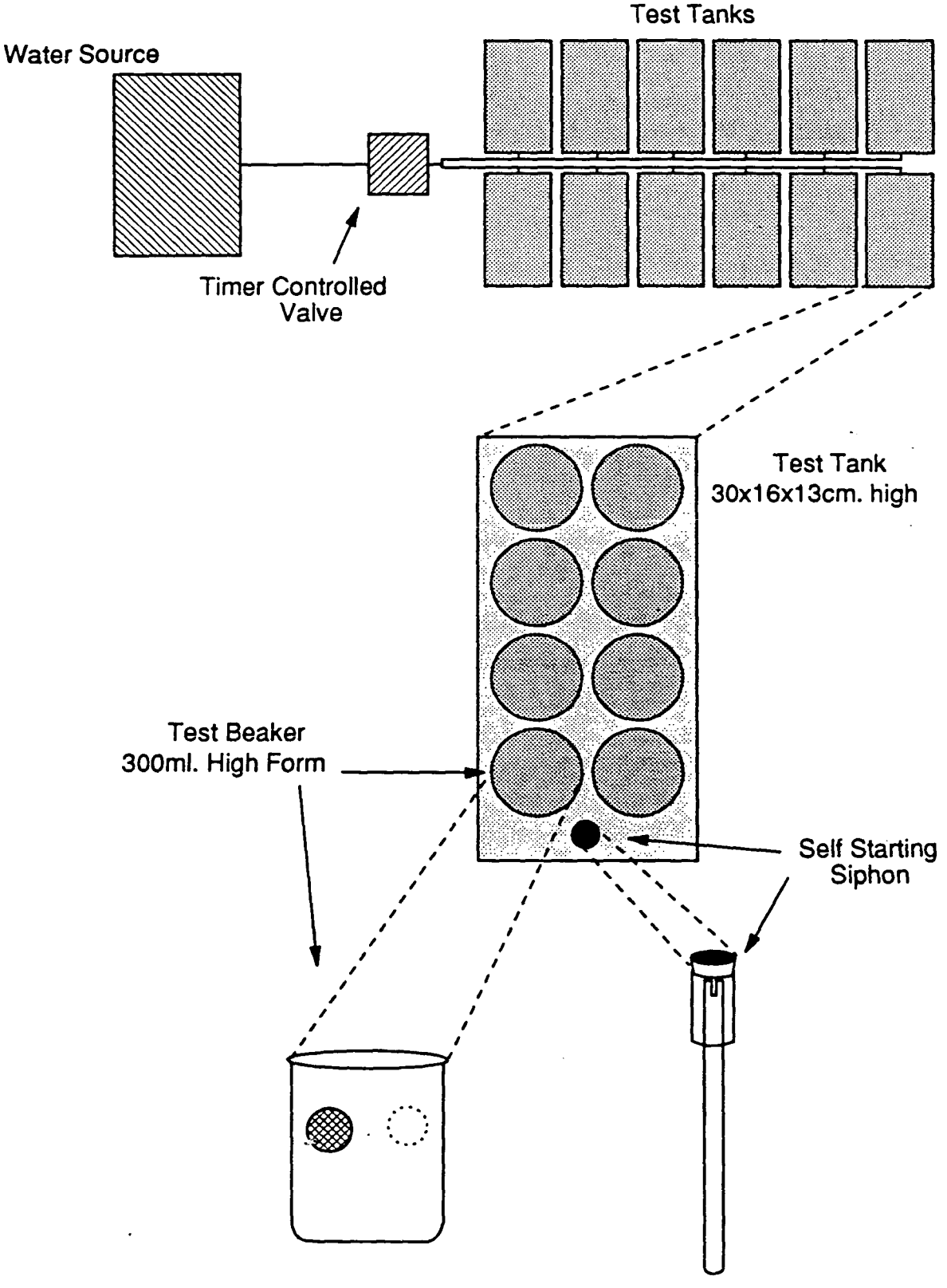

Figure 2. Bioaccumulation of metals (cadmium, nickel) by Lumbriculus variegatus relative to sediment metal concentrations or sediment metal concentrations normalized to acid volatile sulfide (AVS).