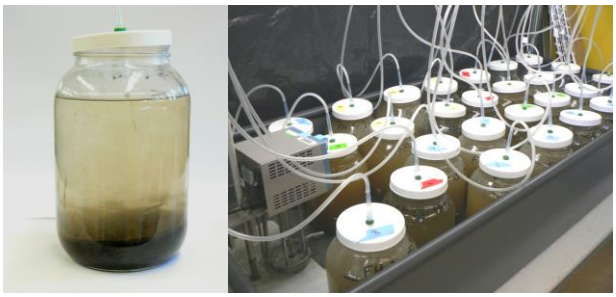
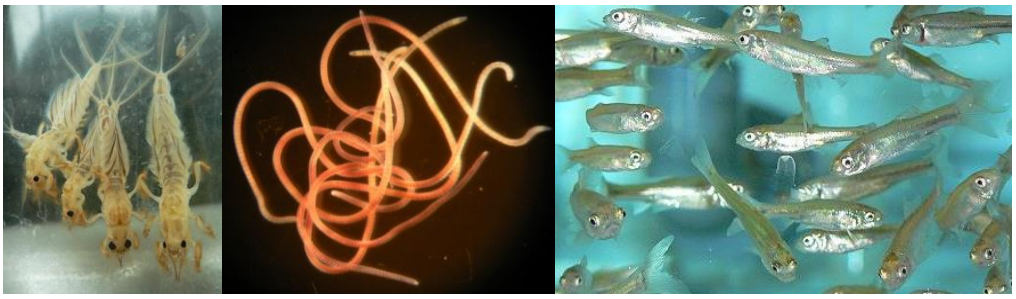
Bioaccumulation of sediment-associated contaminants in freshwater organisms





Bioaccumulation of sediment-associated contaminants in freshwater organisms

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Unpublished method validation information is available upon request from Trudy Watson-Leung or David Poirier.

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Executive Summary

Procedures are described for testing 28-day bioaccumulation potential of chemicals from whole sediments, under static conditions, by three freshwater species: the mayfly, *Hexagenia spp.;* the oligochaete, *Lumbriculus variegatus* and the Fathead Minnow, *Pimephales promelas*. The primary goal in the development of this method was to create a robust, cost-effective standardized laboratory bioaccumulation test for use in site-specific ecological risk assessments. The method however, may easily be applied to new and emerging contaminants of concern, environmental monitoring programs in various jurisdictions, and adapted as necessary to other test organisms or research questions. In this method, only the fathead minnow is fed over the duration of the test. The organic carbon content of the sediment is standardized to a 1:27 sediment total organic carbon (TOC) to organism dry weight ratio, to ensure that the invertebrates have enough organic carbon on which to feed over the duration of the test and no additional food is required. This method has been validated with freshwater sediments collected from the field but may be adapted for use with sediments spiked with compounds in the laboratory. The primary endpoint for the test is whole-body concentration of the contaminants of potential concern (COPCs) and supporting endpoints include lipid content, survival (*Hexagenia spp.* and fathead minnow only) and growth (% change in body weight). Procedures are primarily described for determining COPC concentrations on unpurged animals but guidance is given on gut-purging, should that be of interest to the researcher.

An inter-laboratory study (ILS) of this method was under-taken in 2014 as a final step to validate this method. Control and test field-collected sediments were subsampled and sent to six experienced laboratories for testing of bioaccumulation of arsenic by *Hexagenia* spp. and polychlorinated biphenyls by fathead minnows and to seven laboratories to assess inter-laboratory variability in bioaccumulation of polycyclic aromatic hydrocarbons by *L. variegatus.* All laboratories followed the method as described in this document using their facilities and dilution water. Unpurged organisms were submitted to the MOECC laboratory for analysis. The results of the *Hexagenia* spp. ILS determined that the inter-laboratory variability for all laboratories that met the minimum 80% control survival criterion was 24% and there was no significant difference between laboratories (Watson-Leung et al. 2016). Results of the *L. variegatus* and fathead minnow ILS results were preliminary as of the time of publication of this method.

Foreword

Contamination of sediment in freshwater ecosystems, due to the historical and current release of persistent and toxic substances, is considered a major environmental concern for aquatic ecosystems. The physicochemical properties and persistent nature of many contaminants of potential concern (e.g., PCBs, dioxins and furans, DDT, and mercury) has resulted in their accumulation within sediment. In addition to direct toxicity to benthic organisms, these substances can migrate up the food chain to fish-eating birds, wildlife, and humans through the processes of bioaccumulation and biomagnification. While point source emissions to water and the atmosphere have been dramatically reduced and the production and use of some of these substances has been banned, non-point sources remain and the sediment now serves as a source of many of these contaminants to surface water and aquatic organisms. In addition to the physicochemical characterization of sediment, toxicity testing, and field studies to assess the benthic community, an assessment of the biomagnification potential of contaminants is now part of the decision-making framework for contaminated sediments in Ontario (OMOE 2008a) and elsewhere. Biomagnification potential can be estimated using fugacity based models coupled with food web models and/or estimated from bioaccumulation observed in aquatic organisms in the laboratory (under controlled exposure conditions) or in the field (under naturally occurring exposure conditions). Laboratory assessments using this standard test method support detailed quantitative site-specific effects assessment in environmental risk assessment by determining the bioavailability of these contaminants and providing empirical tissue residue concentrations that can be used for subsequent food-web modeling.

The types of projects supported by this method primarily include sediment assessment and monitoring for sites with known contaminant concerns (e.g., Great Lakes Areas of Concern), spills sites, and evaluation of the effectiveness of remediation procedures, but may also include the assessment of biomagnification potential of new and emerging compounds in field collected or spiked sediments.

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Chapter 1: Terminology, Introduction and Rationale

1.1 Terminology

Note: All definitions are given in the context of this report and might not be appropriate in another context.

Definitions reflect or were modified from those found in:

American Society for Testing and Materials (2010). Standard Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates. Annual Book of ASTM Standards, vol. 11.06. Philadelphia, Pennsylvania, USA. **E 1688-10a:** 53 p.

Environment Canada (1997). Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*. Environmental Protection Series. Ottawa, Ontario, Canada. **EPS 1/RM/33:** 117 p.

United States Environmental Protection Agency (2000). Bioaccumulation Testing and Interpretation for the Purpose of Sediment Quality Assessment: Status and Needs. Washington, DC, USA. **823-R-00-001:** 111 p.

Grammatical terms

Must is used as an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean “is (are) allowed to”.

Can is used to mean “is (are) able to”.

Might is used to express the possibility that something could exist or happen.

General technical terms

Acclimation is physiological adjustment to a particular level of one of more environmental factors such as temperature.

Compositing is the act of combining separate samples (biota, sediment, water) into a single sample.

Conductivity is the numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentration of ions in solutions, their valence and mobility, and the solution’s temperature. Conductivity is reported as micromhos per centimeter (µmhos/cm) or millisiemens per metre (mS/m); 1 ms/m = 10 µmhos/cm.

Flow-through describes apparatus or tests in which solutions or overlying water in culture or test chambers are/is renewed continuously by the constant inflow of fresh solution.

Gut purging is the act of voiding of sediment contained in the gut.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measuring of certain biological or water quality variables, or the collection and testing of samples of sediment for toxicity or bioaccumulation tests.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree of intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24 hour (h) day.

Static describes tests in which test solutions or overlying water are not renewed during the test.

Terms for test material or substances

Chemical is, in this report, any element, compound, formulation, or mixture of a substance that might be mixed with, deposited in, or found in association with sediment or water.

Clean sediment is sediment that does not contain concentrations of any substance(s) causing discernable distress to the test organisms or reducing their survival or growth during the test.

Contaminated sediment is sediment containing chemical substances at concentrations that pose a known or potential threat to environmental or human health.

Control is a treatment in a laboratory investigation of study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific conditions being studied. In an aquatic toxicity or bioaccumulation test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no added test material or substance. The control is used to determine the absence of measurable toxicity/bioaccumulation due to basic test conditions (e.g., temperature, health of organisms, effects of handling, control sediment or water). (See also negative control sediment and positive control sediment).

Control/dilution water is the water used for preparing a series of concentrations of a test chemical, or that used as overlying water in a sediment test or as control water in a water-only test with a reference toxicant. Control/dilution water is frequently identical to the culture and the test (overlying) water.

Control charting is a graphical statistical tool. It is used to monitor method performance, document method uncertainty and detect trends that could impact data quality. Control charting can be used to identify tools for continual improvement in method performance and data quality. Control charting in a toxicity/bioaccumulation testing laboratory must

include charting of the LC50 of organism response to a chosen reference toxicant over time to assess organism health, staff training and system performance. Other uses of control charts include monitoring of culture health parameters (e.g. culture organism lipid content or contaminant concentration), water quality (e.g. chlorine) and organism survival in negative control sediments. See Appendix D.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

Deionized water is water that had been purified by passing it through resin columns or a reverse osmosis system, for the purpose of removing ions such as calcium and magnesium.

Distilled water is a water that had been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Negative control sediment is a clean sediment not containing concentrations of one or more contaminants that could affect the survival, growth, or behaviour of the test organisms. Negative control sediment might be natural sediment from an uncontaminated site, or formulated sediment. The sediment must contain no added test material or substance, and must enable acceptable survival of test organisms during the test. The use of negative control sediment provides a basis for interpreting data derived from toxicity and bioaccumulation tests using sediment(s), and also provides a base sediment for spiking procedures.

Overlying water is water placed over sediment in a test chamber.

Pore water is the water occupying space between sediment or soil particles; also called interstitial water.

Positive control sediment is sediment known to produce a defined effect on the test organisms. A positive control can be an aged spiked field collected or artificial sediment or a field collected sediment with known contamination. Due to issues associated with creating artificial sediments acceptable to the test species, difficulties with spiking sediment, and variability, mixture contamination and potential instability of field collected sediments, positive control sediments are not generally included in sediment toxicity or bioaccumulation tests. However, positive control sediment exposures could be valuable in the assessment of organism health and sensitivity and in the evaluation of the reproducibility (precision) of test data with time.

Reconstituted water is high purity deionized or glass distilled water to which reagent grade chemicals have been added to obtain a desired pH, alkalinity, and hardness characteristics. The resultant synthetic water should be free from contaminants.

Reference sediment is a field-collected sample of sediment that represents “local conditions”. This sediment is presumably clean sediment, selected for properties (e.g., particle size, total organic content) representing sediment conditions that closely match those of the sample(s) of test sediment except for the degree of chemical contaminants. It is often selected from a site uninfluenced by the source(s) of contamination, but within the general vicinity of the sites where samples of test sediment are collected. Sediments that represent “local conditions” may not necessarily be clean and may represent the

background level of contamination associated with aerial deposition or the influence of non-point sources. Comparison to such sites can be useful to identify sites where clean up action might not be useful, because the site is likely to be re-contaminated.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organism in order to establish confidence in the toxicity data obtained from a test material or substance. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision of the reliability of results obtained by the laboratory for that chemical.

Reference toxicity test is a test conducted using a reference toxicant in conjunction with a sediment toxicity or bioaccumulation test, to assess the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect. A reference toxicity test is most often performed in the absence of sediment (i.e., as a water-only test) although it can also be conducted as a spiked sediment test.

Sediment is natural particulate material, which has been transported and deposited in water and usually lies below water. The term can also describe a substrate that has been experimentally prepared (formulated) using selected particulate material and within which the test organisms can burrow.

Solid-phase sediment (also called whole sediment) is the intact sediment used to expose the test organisms, not a form or derivative of the sediment such as pore water or resuspended sediment.

Spiked sediment is any sediment (clean or contaminated) to which a test substance or material such as a chemical, a mixture of chemicals, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the sediment.

Stock solution is a concentrated solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water to prepare the required strength of test solutions.

Substance is a particular kind of material having more or less uniform properties.

Test sediment is a field-collected sample of solid-phase sediment, taken from a site thought to be contaminated with one or more chemicals, and intended for use in a toxicity or bioaccumulation test. In some instances, the term also applies to any sediment sample or mixture of spiked sediment (including negative control and reference sediment) used in the test.

Test water is the water placed over the layer of sediment in the test chambers (i.e., overlying water). It also denotes the water used to manipulate the sediment, if necessary (e.g., for preparing formulated sediment or mixtures of spiked sediment, or for wet sieving) and that used as control/dilution water for water-only tests with reference toxicants.

Whole sediment is also called solid-phase sediment (see above).

Statistical and toxicological terms

Bioaccumulation is the net accumulation of a chemical by an organism through all routes of exposure (including respiration, ingestion, or direct contact with contaminated water, sediment, or pore water).

Bioaccumulation factor (BAF)[1] is the ratio of a substance's concentration in tissue of an aquatic organism to its concentration in the ambient water, in situations where both the organism and its food are exposed.

Bioaccumulation potential is the qualitative assessment of whether a contaminant in a particular sediment is bioavailable.

Bioavailable chemicals are those in the state of being potentially available for biological uptake by an aquatic organism when that organism is processing or encountering a given environmental medium (e.g., the chemicals that can be extracted by the gills from the water as it passes through the respiratory cavity or the chemicals that are absorbed by internal membranes as the organism moves through or ingests sediment). In water, a chemical can exist in two basic forms that affect availability to organisms: (1) dissolved or (2) sorbed to biotic or abiotic components and suspended in the water column or deposited on the bottom.

Bioconcentration is the net accumulation of a chemical directly from water into an aquatic organism.

Bioconcentration factor (BCF) is the ratio of a substance's concentration in the tissue of an aquatic organism to its concentration in the ambient water, in situations where the organism is exposed through water only.

Biomagnification is the uptake of bioaccumulative organic contaminants via dietary uptake (bioconcentration is a possible secondary route of exposure) through a food chain resulting in increasing tissue concentrations of the bioaccumulative chemical up through three or more trophic levels. The term implies an efficient transfer of chemical from food to consumer, so that residue concentrations increase systematically from one trophic level to the next.

Biota-sediment accumulation factor (BSAF)[1] is the ratio of a substance's concentration in the tissue of an aquatic organism to its concentration in the sediment. For organic

[1] Note that the definitions provided here are refined from what is provided by the USEPA. USEPA (2000a) provides the same definitions of BAF and BSAF, however a calculation of BAF_w for accumulation from water and BAF_s for accumulation from sediment are also provided within the body of the document. ASTM (2010) defines BAF as the ratio of tissue residue to sediment concentration at steady-state and identifies BAF as analogous to BCF which is used to predict tissue residues from water concentrations. USEPA (2000b) and ASTM (2010) define BSAF as the ratio of tissue residue to source concentration (e.g., sediment at steady state normalized to lipid and sediment organic carbon) and state that this formula is not appropriate for inorganic substances (e.g. metals) since the lipid normalized relationship does not apply for the accumulation of metals.

contaminants, concentrations are normalized for lipid content and total organic carbon for tissue and sediment, respectively.

Bioturbation is the act of resuspending contaminated fluid or sediment particles into the water column through turbulent activities of biota.

Coefficient of variation (CV) is the standard deviation (SD) of a set of data divided by the mean, expressed as a percentage. It is calculated as: CV (%) = 100 x SD ÷ mean.

Contaminants of potential concern (COPC) are chemicals identified as a potential concern in sediment quality assessments because of their potential to accumulate in the tissue of organisms through any route of exposure.

Depuration is the loss of a substance from an organism as a result of any active (e.g., metabolic breakdown) or passive process when the organism is placed into an uncontaminated environment. Contrast with elimination.

Elimination is the general term for the loss of a substance from an organism that occurs by any active or passive process. The term is applicable in either a contaminated environment (e.g., occurring simultaneously with uptake) or a clean environment. Contrast with depuration.

Endpoint means the variable(s) (i.e., time, reaction of the organisms, etc.) that indicate(s) the termination of a test, and also means the measurement(s) or derived value(s) that characterize the results of the test (e.g., LC50, BSAF).

Equilibrium partitioning refers to the assumption that a thermodynamic equilibrium exists between the chemical concentrations in sediment, pore water, and benthic organisms.

Equilibrium partitioning bioaccumulation models are based on equilibrium partitioning of a neutral organic compound among organism lipids and sediment carbon.

Food chain model is a mathematical model that estimates the quantitative transfer of a chemical(s) through the different trophic levels of the food chain. These models vary in complexity, can contain many state variables and parameters, and consider the movement of a chemical through a food chain consisting of one or more trophic levels. These models are typically used with toxic, nonselective, and bioaccumulative chemicals that can affect the entire structure of an ecosystem.

In situ means in the natural or original position; the use of field and natural conditions rather than the standardized conditions of laboratory experiments.

LC50 is the median lethal concentration, i.e., the concentration of substance or material in sediment or water estimated to be lethal to 50% of the test organisms. The LC50 and 95% confidence limits are usually derived by statistical analysis of mortalities of five or more test concentrations, after a fixed period of exposure.

Lethal means causing death by direct action. Death of organisms is defined as the cessation of all visible signs of movement or activity indicating life.

Precision refers to the closeness of reproduced measurements of the same quantity to each other. It describes the degree of certainty around a result.

Replicate refers to a single test chamber containing a prescribed number of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). A replicate is an independent experimental unit; therefore, any transfer of organisms or test material from one replicate to another would invalidate the test.

Steady-state is a ‘constant” tissue residue resulting from the balance of the flux of compound into and out of the organism, determined operationally by no statistical difference in three consecutive sampling periods.

Sublethal means detrimental to the organism, but below the level that directly causes death within the test period.

Sublethal effect is an adverse effect on an organism, below the level that directly causes death within the test period.

Toxicity is the inherent potential or capacity of a substance or material to cause adverse effect(s) on living organisms. The effects could be lethal or sublethal.

Toxicity test is a procedure for determining the effect of a substance or material on a group of selected organisms under defined conditions. An aquatic toxicity test usually measures: (a) the proportion of organisms affected (quantal); and/or (b) the degree of effect shown (quantitative or graded) after exposure to a specific test substance of material (e.g., a sample of sediment) or mixture thereof (e.g., a chemical/sediment mixture).

Tissue residue is the concentration of contaminant in an organism’s tissue.

Trophic levels are the different feeding relationships in an ecosystem that determine the route of energy flow and pattern of chemical cycling.

Trophic level transfer is the transfer of chemical from food to consumer.

Acronyms of institutions/agencies

ASTM - American Society for Testing and Materials

EC - Environment Canada

ISO - International Organization for Standardization

MOECC - Ontario Ministry of the Environment and Climate Change (prior to 2015 known as Ontario Ministry of the Environment (OMOE))

USEPA - United States Environmental Protection Agency

Glossary of abbreviations

Hg – mercury

As – arsenic

PCB – polychlorinated biphenyl

dl-PCB – dioxin-like polychlorinated biphenyl

DDT – dichlorodiphenyltrichloroethane

DDE – dichlorodiphenyldichloroethylene

PBDE – polybrominated diphenyl ether

PAH – polycyclic aromatic hydrocarbon

PCDD/F - polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF)

Mention of trade names of products, suppliers or other companies does not indicate endorsement by the Ontario Ministry of the Environment and Climate Change.

1.2 Introduction

1.2.1 Background

Contamination of sediment in freshwater ecosystems, due to the historical and present release of persistent and toxic substances, is considered a major environmental concern for aquatic ecosystems. The physicochemical properties and persistent nature of many contaminants of potential concern (e.g., polychlorinated biphenyl (PCB)s, dioxins and furans, dichlorodiphenyltrichloroethane (DDT), and organic mercury) has resulted in their accumulation within sediment. Not only may these substances be toxic to benthic organisms, but many are transferred from the sediment into benthic organisms and fish and further up the food chain to fish-eating birds, wildlife, and humans through the processes of bioaccumulation and biomagnification. Although industry has reduced emissions to water and the atmosphere based on environmental regulations and the production and use of some substances has been banned, the sediment still serves as a source of many of these historical contaminants. In addition to the physicochemical characterization of sediment, toxicity testing, and benthic surveys, an assessment of the biomagnification potential of sediment-bound contaminants is now part of the decision-making framework for contaminated sediments in Ontario (OMOE 2008a) and elsewhere. Laboratory assessments using this standard test method support detailed quantitative site-specific effects assessment in environmental risk assessment by determining the bioavailability of contaminants that may biomagnify.

As the demand for robust and cost-effective tools to use in ecological risk assessment increases, there is also a need to standardize procedures for assessing the bioaccumulation of contaminants from sediment. Field collections and studies are the most ecologically relevant approaches to assessing bioaccumulation. However, the use of mobile organisms, such as fish, may prove difficult to delineate zones of contamination and the sampling of benthic organisms is often limited due to the workload constraints of obtaining sufficient biomass. Caging of organisms in the field provides a balance between experimental control and ecological relevance, but these *in situ* studies are still time consuming, labor-intensive, and prone to vandalism, predation, or destruction. Compared to field studies, laboratory methods offer more flexibility with timing and typically reduce workload, cost, and seasonal and spatial variability. They allow control of environmental conditions and standardization of exposure techniques and test species used. They also allow for the assessment of research type questions (i.e., species differences, kinetic uptake, emerging contaminants of concern) or the integration of additional assessment endpoints (e.g., survival, growth, reproduction). Since organisms cannot as easily avoid contaminants in a test system, laboratory methods represent what is potentially a worse case exposure scenario. Laboratory methods cannot replace or replicate field exposures, but are intended to support field methods and/or fill data gaps. Although bioaccumulation data are frequently generated and applied on a site-specific basis, the use of standard methods enables contaminated sites to be compared to each other on a scientifically defensible basis and accurately ranked with respect to priority of clean-up and effectiveness of remediation. Standard methods that have undergone proper method development and validation are likely to

improve the translation of laboratory data to the field, thereby strengthening ecological risk assessments and regulatory decision-making.

As in toxicity testing, no one species may be best suited to assess all possible environmental conditions encountered in routine testing. Different species and taxa may vary in bioaccumulation potential, which may also be influenced by contaminant and sediment type. In regards to both toxicity and bioaccumulation testing with sediment, there is much support in the literature for a multi-species test battery with organisms representing different taxa, trophic levels, and potential routes of exposure (Giesy and Hoke 1989; Burton 1991; Burton et al. 1996; ASTM 2010). Mac and Schmidt (1992) suggest that the most comprehensive assessment of bioaccumulation includes both benthic invertebrates and fish species that have some association with sediment. The American Society for Testing and Materials (ASTM 2010) also suggests that the use of two or more species from different major taxa increases the probability of measuring maximum tissue residues and recommends this for assessing moderate to large discharges or dredging operations. However, an insufficient number of multi-species comparative studies have been conducted to adequately compare the bioaccumulation potential of a variety of species with a range of contaminants (ASTM 2010).

Currently, only laboratory bioaccumulation methods with the oligochaete *Lumbriculus variegatus* are standardized to the point of providing recommendations for specific conditions (i.e., ASTM 2010; United States Environmental Protection Agency-USEPA 2000b) and many researchers have used this species based on these protocols. While general guidance for conducting laboratory bioaccumulation tests with other invertebrates is offered in ASTM (2010), the need to further standardize these methods and identify/develop methods with additional species has been identified as a priority (USEPA 2000a). A bioaccumulation method (Bedard et al. 1992) with fathead minnows has been used by the Ontario Ministry of the Environment and Climate Change (MOECC) for many years. Although this method has been fairly effective, there was a need to re-evaluate and standardize test procedures and expand the method to include the use of invertebrate species that burrow and eat sediment.

A new bioaccumulation method developed for the MOECC is described in this document and is based on the ASTM (2010), USEPA (2000b), and Bedard et al. (1992) methods, with several distinctions. Most notably, this protocol uses a loading density of organisms different than that specified in the other methods and selected based on the results of experiments conducted as part of the method development. In addition, unlike the ASTM (2010) and USEPA (2000b) methods this method is performed under static conditions. This protocol uses the same length of exposure as the ASTM and USEPA methods and ratio of sediment and overlying water used in Bedard et al. (1992), but expands upon the selection of test endpoints and performance-based validity criteria. After a review of the existing literature (Van Geest et al. 2010), selection of test species, and testing of data gaps regarding exposure techniques, this bioaccumulation method was established for use in definitive testing. This was followed by an assessment of the bioaccumulation potential between test species (Van Geest et al. 2011c). Validation of these methods involved the comparison of 2007/08 sampling and laboratory data with available field data from nine corresponding sites in Ontario (Van Geest et al. 2011d). All of this work was conducted while operating under the quality management system of

the Laboratory Services Branch of the MOECC (OMOE 2008b) This system meets all requirements of ISO17025-2005 General requirements fro the competence of testing and calibration laboratories (International Organization for Standardization – ISO 2005).

This test method is based on:

American Society for Testing and Materials. 2010. Standard Guide for Determination of the Bioaccumulation of Sediment-Associated Contaminants by Benthic Invertebrates. Annual Book of ASTM Standards, vol. 11.06. Philadelphia, Pennsylvania, United States. **E 1688-10a:** 57 p.

Bedard, D., A. Hayton and D. Persaud. 1992. Laboratory Sediment Biological Testing Protocol. Ontario Ministry of the Environment, Queen's Printer for Ontario**:** 26 p.

United States Environmental Protection Agency. 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates. Duluth, Minnesota, United States. **600/R-99/064:** 212 p.

1.2.2 Principal of the method

In laboratory assessments of bioaccumulation, test organisms are placed in chambers containing sediment and overlying water and allowed to accumulate contaminants from the sediment for a chosen period of time. Field-collected organisms from reference locations may be used, but issues may arise with the transfer, maintenance in the laboratory, background levels of contaminants, and life stage of these organisms. The use of laboratory-reared organisms ensures the availability of a particular species, of a specific age or size, with a known exposure history.

The purpose of this method is to measure the bioaccumulation of contaminants from sediment into freshwater organisms under laboratory conditions. It was designed to assess field-contaminated sediments by determining how bioavailable the contaminants are and making a best estimate of steady-state concentrations in tissues that can be achieved in both a timely and economical manner. These data are used in characterizing sediment quality, modeling trophic transfer, and as a component in the assessment of environmental risk posed at contaminated sites in Ontario. Since the whole organism is typically consumed by fish, waterfowl or other predators, this method was validated primarily by using whole-body concentrations on unpurged organisms.

It should be clarified that bioaccumulation assessment using this method does provide a measure of effect or risk. It is a measure of the bioavailability of a contaminant of potential concern (COPC) and can provide an indication of the hazard posed to higher trophic level consumers. It may be of interest to adapt this method for sediment quality assessments wishing to use the tissue residue approach to toxicity assessment (Jarvinen et al. 1996), however this approach was not validated during method development.

Based on the objectives of the study it may or may not be desirable to purge the organisms of their gut contents prior to test completion. For example, COPCs in edible tissue are better indicators of human health risk (Ingersoll and MacDonald 2002).

Sediment associated contaminants from gut contents of organisms may lead to over or under estimation of tissue concentration and purging can reduce this bias (Van Geest *et al.* 2011c). However, depuration and metabolism of compounds (particularly low molecular weight compounds which are quickly depurated) can occur during gut purging and impact the estimate of tissue concentrations (Van Geest *et al.* 2011c; ASTM 2010). A side by side comparison of whole-body concentrations in purged vs. unpurged organisms was conducted on four field collected contaminated sediments (mercury (Hg), polychlorinated biphenyl (PCB), dioxin-like polychlorinated biphenyl (dl-PCB), dichlorodiphenyltrichloroethane (DDT) and polyaromatic hydrocarbon (PAH)) during the validation of this method with the three species (Van Geest *et al.* 2011c). Based on the results of this study it was determined that for studies interested in risk assessment, field-to-lab comparisons and food web modeling, the extra effort involved in purging organisms may not be necessary. The procedures to conduct a purge in water have been included for situations where it is considered appropriate (e.g., uptake and elimination kinetics, spiked sediment, research-type investigations). Refer to section 3.3.5.5 for more details.

This method offers the use of two invertebrate species, the oligochaete *Lumbriculus variegatus* and the mayfly nymph *Hexagenia* spp. and one fish species, the fathead minnow *Pimephales promelas*. These species were chosen to reflect differences in taxa, trophic level, and bioaccumulation potential (e.g., routes of exposure, metabolic capabilities, etc), in addition to being ecologically relevant in North American ecosystems. The oligochaete and mayfly species allow for the evaluation potential dietary uptake by bottom-feeding fish or sediment-probing birds while the mayfly is also an emergent insect and may allow for investigation of potential uptake by terrestrial predators such as birds and bats. In situations where the receptor(s) of concern is/are fish-eating birds and/or mammals, the minnow would provide information about risk of dietary uptake. This suite of species should provide what is often considered the missing link when assessing the movement of contaminants between sediment and fish and other wildlife. Our goal is to provide a method that is robust, practical, and effective and may easily be applied to new and emerging contaminants of concern, environmental monitoring programs in various jurisdictions, and adapted as necessary to other test organisms (e.g. mussels) or research questions.

In the context of a sediment assessment framework (OMOE 2008a; COA 2008) laboratory bioaccumulation tests are conducted once biomagnification is identified as a potential concern. Detailed quantitative testing (step 5 and 6) assessment of site-specific bioaccumulation is of interest for projects for which site sediment chemistry concentrations or previous toxicity testing has indicated that toxicity is not of concern but biomagnification is of potential concern. The types of projects supported by this method primarily include sediment assessment and monitoring for sites with known contaminant concerns (e.g., Areas of Concern), spills sites, and evaluation of the effectiveness of remediation procedures, but may also include lakefilling/shoreline creation projects (placement of fill material in water) and the assessment of dredged material for disposal.

1.3 Rationale for Sediment Bioassessment Methodology

1.3.1 Test methodology

Many of the factors that are related to the experimental design of a method and have the potential to affect the outcome of a bioaccumulation test have been discussed extensively in ASTM (2010). The procedures used in this method were selected in an attempt to balance scientific, practical, and cost considerations, while providing the most precise data for the intended applications of this method. Many of the exposure conditions and techniques were based on best scientific practice (as reviewed in the literature by Van Geest et al. 2010c) and experimental evidence from research conducted as part of the development and standardization of this method. All supporting information that has not been published is available upon request from the Ministry of the Environment and Climate Change (MOECC).

This bioaccumulation protocol uses static[2], single-species tests with whole sediment. Negative control and test sediments are placed in glass jars and test water is added to achieve a 1:4 (volume/volume) ratio of sediment to overlying water[3]. Overlying water is aerated throughout the test to maintain acceptable water quality (e.g., dissolved oxygen (DO) >2.5 mg/L, unionized ammonia <0.2 milligrams per litre (mg/L)). Cultured or reared test organisms are added to the exposure chambers at a density of ~27:1 ratio of sediment total organic carbon to organism dry weight[4]. Depending on the objectives of

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[2] The ASTM (2010) and USEPA (2000b) bioaccumulation methods recommend conducting whole-sediment tests with flow-through or renewal of overlying water (i.e., 2 volume additions/day) to minimize degradation of water quality. However, this option relies heavily on the proper function of water distribution systems or then requires more work if water renewal is done manually. From a practical standpoint, static tests provide the greatest flexibility with test set up. As a closed system, static tests reflect a potentially worse-case exposure scenario. Static tests with aeration and a sufficient proportion of overlying water have been used successfully in OMOE sediment toxicity tests and in development of this bioaccumulation method.

[3] No recommendations of a sediment to water ratio is made in the ASTM (2010) and USEPA (2000b) bioaccumulation methods. A 1:4 ratio is used in OMOE sediment methods (Bedard et al. 1992), as well as standard marine methods (ASTM 2008). Work has been done examining the effects of different sediment to water ratios on toxicity to *Hyalella azteca* (by Borgmann and Norwood 1999; Watson-Leung and Nowierski 2005), but has not been related to bioaccumulation testing. In a review of published studies 30% of studies used a ratio between 1:3 and 1:5 however ratios ranged from 1:1 to 1:1000 and the ratio used could not be determined from 45% of studies (Van Geest et al. 2010c).

[4] The ASTM (2010) and USEPA (2000b) bioaccumulation methods recommend maintaining a minimum of a 50:1 ratio of sediment TOC to organism dry weight to minimize the depletion of contaminants in the sediment. These methods also standardize to a constant volume. Loading density of organisms was one of the most noted inconsistencies in the literature (Van Geest et al. 2010c). Other loading densities have been used by different researchers, including a 27:1 ratio of TOC to organism dry weight by Leppanen and Kukkonen (2004). Further support of standardizing organism density to sediment TOC and the selection of a 27:1 ratio is based on experimentation in development of this method (Van Geest et al. 2010b). This bioaccumulation method is based on the understanding that TOC is the limiting factor in the ability of organisms to bioaccumulate contaminants from sediment exposure. The greater the concentration of TOC in a sediment sample, the greater the capacity exists for contaminants to bind to the sediment. Therefore this bioaccumulation method was standardized for TOC concentration to minimize the influence of the variation of TOC from sample to sample and standardize the amount of contaminants available to the organisms. The decision was made not to standardize this method for both

the test and expected variability in COPCs (refer to section 4.1.2.1) tests are run with a minimum of three replicates per test sediment[5]. The tests are carried out at 23 ± 2 degrees Celsius (°C), with a 16:8 hour (h) light:dark photoperiod for 28 days[6]. Organisms may or may not be purged at the end of the test, depending on the objectives of the study[7]. This protocol offers the use of three test species from different taxa and trophic levels to reflect differences in bioaccumulation potential and application of data (as recommended by Mac and Schmidt 1992; ASTM 2010).

1.3.2 Selection of Test Organisms

The three freshwater organisms selected for this method were the oligochaete worm *Lumbriculus variegatus*, the mayfly nymph *Hexagenia* spp., and the fathead minnow *Pimephales promelas*[8].

Lumbriculus variegatus has been used primarily by the USEPA for assessing bioaccumulation potential in dredged sediments, but has also been used extensively in research-based bioaccumulation methods. This species is tolerant of a range of sediment characteristics, and burrows into and ingests sediment. Since organisms are small (5-12 milligram (mg)), large groups of organisms are required to attain sufficient biomass for chemical analysis. They reproduce asexually through fragmentation rather quickly, which is advantageous for culturing; however, this means that sediment and contaminant uptake may not take place continuously throughout a test while portions of the body are regenerated (Leppänen and Kukkonen 1997; White *et al.* 1987).

The mayfly nymph *Hexagenia* spp. (cultured from mixed culture of eggs from the ecologically and taxonomically similar species *H. limbata* and *H. rigida*)[9] reflects an

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TOC:organism dry weight and sediment volume in an effort to address the pivotal role that TOC plays in contaminant loading of sediments and to standardize organism exposure.

[5] This method, designed to be used primarily by "production labs" was validated using three laboratory replicates. The USEPA recommends a minimum of five replicates per treatment for bioaccumulation testing however recommends the inclusion of as many replicates as economically and logistically possible (USEPA 2000b).

[6] A 28-day exposure has been suggested as a standard as this typically results in tissue residues within 80% of steady-state (ASTM 2010). The majority of studies assessing field-contaminated sediment in the literature were also of this duration (Van Geest et al. 2010c).

[7] Purging of organisms did not improve the estimate (i.e., reduce variability) of tissue concentrations of organic contaminants in the three test species (Van Geest et al. 2011c). The potential error associated with gut contents appears be of greater concern for metals (Brooke et al. 1996), however this remains to be investigated with respect to metals with potential to biomagnify (i.e., organic mercury) and the test species of this method.

[8] A variety of freshwater species have been rated for bioaccumulation testing by Ingersoll et al. (1995) and ASTM (2010) and their use is discussed in Van Geest et al. (2010c).

[9] Due to advances in genomics technologies it is now possible to taxonomically identify *Hexagenia* adults and nymphs to species (Elderkin et al. 2012). All method development work for this method was performed with eggs collected from a mix of imagoes of *H. rigida* and *H. limbata* emerging at the same time and location. The proportion of these species in the population emerging in the western basin of Lake Erie varies from year to year (Corkum 2010). *Hexagenia rigida* did not hatch after egg storage at 8°C for a year while *H. limbata* nymphs had hatching success of 44.6 % under the same storage

appropriate route of exposure since it burrows into and ingests sediment. *Hexagenia* spp. are currently used by the MOECC and elsewhere in sediment toxicity tests assessing survival and growth (ASTM 2005; Bedard et al. 1992). In addition to providing adequate biomass, nymphs naturally inhabit the sediment for a year or more and are thus suited for long-term exposures. Both nymph and adult stages of this insect represent an important food source to fish and other wildlife. *Hexagenia* spp. nymphs have been shown to demonstrate a preference for finer grained sediments in which burrows can be maintained (Wright and Mattice 1981). Sampling of organisms in the field has shown that *Hexagenia* spp. nymphs have higher body burdens of contaminants (e.g., PAHs and PCBs) than dreissenid mussels, amphipods, and crayfish (Gewurtz et al. 2000).

The juvenile fathead minnow, *P. promelas*, has been used by the MOECC in an 'in-house' bioaccumulation method (Bedard et al. 1992) for many years to identify zones of potential exposure in contaminated sites. This species, in particular, has been extensively used in toxicity testing and large databases exist regarding its sensitivity to a variety of contaminants and endpoints (e.g., USEPA ECOTOX database). It is also one of the fish species listed in the ASTM (2003) guide for bioconcentration tests. While fish do not interact with sediment to the same extent as burrowing invertebrates they do offer some unique advantages as suggested by Mac and Schmidt (1992). Fish often resuspend the sediment which can increase the exposure of fish to contaminants (McCarthy *et al.* 2003; Spacie *et al.* 1995). They may reflect several routes of exposure by accumulating contaminants dissolved in the water (through gills and skin), from sediment particles (surface of gill), and through the ingestion of food and sediment. Fish provide adequate tissue mass with high lipid content, are easily collected or caged in the field for comparison, and ecologically important as food items of higher level consumers. It should be noted however, that the addition of food to the fathead minnow test may reduce contaminant bioavailability.

Although they may be able to tolerate a wider range of conditions, maintaining the following water quality parameters will ensure there is no confounding stress caused to fathead minnows, *Hexagenia spp.* or *L. variegatus*: unionized ammonia maintained below 0.2 mg/L (Thurston et al. 1986; OMOE 1999), dissolved oxygen maintained above 60% saturation (USEPA 1986; OMOE 1979), pH between 6.5 and 8.5 (OMOE 1979), alkalinity reduced no less than 25% from culture water (OMOE 1979) and hardness no less than 50% of culture water hardness (USEPA 2000b).

1.3.3 Parameters measured and selection of biological endpoints

This method measures survival (%), growth (% change in average or total biomass), lipid content (where appropriate), and bioaccumulation in the exposed test species. Bioaccumulation is reported in terms of absolute tissue concentration and as a biota-sediment accumulation factor (BSAF), which represents bioaccumulation relative to the

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conditions (Bustos and Corkum 2013). Green et al. (2013) postulated that the timing and success of egg hatching of *H. rigida* explains their early recolonization of Lake Erie while the shift in dominance to *H. limbata* is explained by the ability of *H. limbata* eggs to overwinter in sediments and possible tolerance of nymphs to episodic hypoxia.

concentration of the contaminant(s) of potential concern (COPC) in sediment. Contaminants known to accumulate a rate greater than that at which they are lost, include but are not limited to PAHs, PCBs, dioxin-like PCBs, polychlorinated dibenzo-p-dioxins and dibenzofurans, DDT and metabolites, perfluorinated compounds, halogenated (bromo-, chloro-) PAHs, and arsenic. Accumulation of these compounds has been documented using this laboratory method (Van Geest et al 2011c)[10]. This method may also be used to evaluate sediment contaminated with other known or emerging persistent organic pollutants (e.g., mercury, brominated flame retardants).

Bioaccumulation is typically the main ecologically significant endpoint investigated in laboratory bioaccumulation methods because other tests with more sensitive species and endpoints have been designed to assess the toxicity of sediment (e.g., Environment Canada – EC 1997 and 2013). Although the species used in bioaccumulation methods are often selected for their relative tolerance of pollution, many field-contaminated sediments that are of environmental concern still have the potential to elicit moderate to high toxicity to these organisms. Although mortality occurring under laboratory conditions can be presumed to reflect what may occur under environmental conditions, this has the potential to affect estimates of bioaccumulation via altered behavior, feeding or loss of biomass associated with the stress, acclimation (or compensation), and/or avoidance of the contaminants. High mortality resulting in the loss of biomass for chemical analysis has prompted the recommendation to conduct a short-term test to screen the toxicity of any sediment prior to use in a bioaccumulation test (ASTM 2010; USEPA 2000b and see section 3.2 in this document).

Even if toxicity is not observed in this preliminary test, survival and growth are still important to measure in a definitive bioaccumulation test for quality control purposes and may assist in the interpretation of bioaccumulation data. Mortality or stress occurring in control exposures indicates that the health of test organisms may have been compromised initially or that the test system was contaminated. Ideally, growth should occur over the 28-day test, but differences in sediment type (total organic carbon (TOC) and particle size) could make it difficult to include this as a sensitive endpoint for burrowing invertebrates. Growth cannot be considered a sensitive endpoint for fathead minnows due to the life stage and length of exposure used in this method. Survival, growth, and reproduction of *L. variegatus* cannot be separated in a bioaccumulation test due to the large quantity of worms required to achieve sufficient biomass for chemical analyses. Therefore, change in total biomass is measured for this species. In reference or test treatments, mortality and signs of stress, including loss of biomass and avoidance or lack of burrowing in sediment, may alter exposure and therefore bioaccumulation of contaminants.

In tests assessing the bioaccumulation potential of metals it is important to understand whether uptake or depuration of the COPC is well-regulated by the organism as an essential nutrient or due to inducible detoxification mechanisms through metabolism or excretion (Bechtel Jacobs Company LLC. 1998). In such cases whole-organism COPC concentrations will remain constant, independent of the sediment concentrations to which they are exposed. In addition, factors such as lack of induction at low

[10] Unpublished data available upon request from the MOECC.

concentrations and saturation kinetics or toxicity at high concentrations and other such processes can affect the interpretation of bioaccumulation test results for metals (Bechtel Jacobs Company LLC 1998). This method may be valuable as a standardized method for assessments linking body burden to biological effects (e.g. critical body burden/tissue residue approach) or biomagnification potential of regulated metals.

In tests assessing the bioaccumulation of organic contaminants it is important to measure lipid content in the tissue of exposed organisms in addition to the contaminant(s) of concern. Many organic contaminants partition into lipids, therefore an organism with a higher lipid content will likely have a higher total body concentration of the COPCs. Normalizing tissue concentrations of contaminants for lipid content is a common practice to minimize these differences and reduce variability, particularly for comparisons between species, or for comparison with equilibrium partitioning bioaccumulation models.

Measurement of lipid and total biomass can also provide an indication of metabolic health if comparisons are made between organisms at the beginning and end of a test. These comparisons can indicate whether growth has occurred or whether the organisms have entered a state of starvation metabolism (i.e., a decrease in lipid content) due to avoidance or reduced ingestion of contaminated sediment or insufficient nutrients. These changes in the metabolic health of organisms are important to identify as they have the potential to indicate altered exposure and influence the interpretation of results.

In tests where the assessment of the mass balance of contaminants between media (sediment, biota and water) is of interest, the measurement of COPCs in the sediment upon test termination is required. This will be easily achieved with the fathead minnow and, with minimal additional effort to avoid addition of water during test termination, bioaccumulation tests with *Hexagenia* spp. could also be used in mass balance assessments. Due to the amount of additional water required to extract *L. variegatus* from the sediment, the COPCs in sediments saved after test termination will likely be diluted or reduced due to loss of fine materials. Bioaccumulation testing with this organism may therefore be of limited value in mass balance assessments.

Chapter 2: Test Organisms, Test System and Project Planning

2.1 Test Organisms

2.1.1 Species and life stage

Tests are conducted with adult *L. variegatus* from cultures that reproduce through asexual fragmentation. Oligochaetes used in toxicity-screening and reference toxicant tests should have both head and tail segments that are completely developed. Oligochaetes used in definitive bioaccumulation tests are added as a clump of worms (based on required wet weight), for which this recommendation of complete development does not apply due to practical considerations.

Hexagenia spp. hatch from eggs as nymphs and while inhabiting the sediment undergo successive molts until they emerge as winged, short-lived adults. Nymphs are used in

all tests and for bioaccumulation tests nymphs are reared to a size that provides sufficient biomass while not being so large as to likely emerge as adults during the test. The recommended average size for use in the bioaccumulation test is 20-30 mg while nymphs that have begun to show signs of late instar development (i.e., darker, thickened wing pads, larger eyes, lightened colour of eyes) must not be used in testing due to likelihood of emergence. For toxicity-screening tests, organisms between 6 and 40 mg can be used since the test is of shorter duration than the bioaccumulation test, although a smaller range in size is preferred.

With fathead minnows, juveniles (3-6 months, 250-400 mg) that have not shown signs of sexual differentiation are used in tests (refer to section 2.1.3.11). Juvenile fish are those considered post-larval or older and actively feeding, but not sexually mature, spawning, or recently spent (ASTM 2003). This reduces the impact of confounding factors such as differential lipid deposition and metabolism of reproductively mature males and females. The use of juveniles instead of fry represents a life stage in which the fish are less sensitive to the toxic effects of contaminants and can withstand the long-term exposure of the test. Juveniles are also more likely to interact with the sediment than fry due to feeding behavior. This life stage provides fish of a sufficient size to easily attain the sample size of whole fish required for analytical methods.

2.1.2 Source and acclimation

All organisms used in a test must come from the same source. Sources of animals used to establish cultures may come from government or private laboratories or a commercial biological supplier. Fish are best acquired from a source known to have disease-free fish. Just as is required for cultures maintained “in-house”, if purchasing organisms for a test it is important to assess health criteria (see section 2.1.3.12) on these organisms prior to their use in testing.

Taxonomic identification records for organisms should be received with the shipment of organisms. If these are not available, the taxonomy of organisms must be confirmed by sending samples to a qualified taxonomist or for genome analysis.

Organisms should be transported using the source water in which they were cultured, and water should be well oxygenated (90-100% saturation). Temperature should be maintained as close as possible to the culturing temperature and travel time should be minimized. Crowding of organisms should be avoided to minimize stress and depletion of oxygen.

Once organisms are received at the laboratory record the temperature upon arrival, dissolved oxygen (DO), hardness, and the number dead or stressed. Organisms may be held in the water used in transit while any temperature adjustments are made or transferred to well oxygenated culture water adjusted to the temperature of the shipment. Gradual exposure of organisms to culture water is recommended if there is a great difference in the chemistry (e.g., hardness, pH, conductivity) of water in which they were reared[11]. Temperature adjustments should be gradual and should not exceed

[11] It is advisable to add lab water to the transit water in increasing ratios, maintained for 2-3 hours per increment (e.g. 0: 100, 75:25, 50:50, 25:75 and finally 100:0).

a rate of 2°C per day (USEPA 2000b). Water should be aerated gently during this acclimation period.

2.1.3 Culturing

2.1.3.1 General

General guidance for culturing the test organisms in preparation for testing is provided in this section and conditions have been summarized in Table 1. Many of the procedures recommended are based on existing methods published elsewhere. Where they have been established, performance-based or health criteria are used to assess the suitability of organisms for testing (section 2.1.3.12). The relative health of the cultures must also be assessed through concurrent and/or routine tests (i.e., on a quarterly basis as a minimum and on each new batch of organisms) with a reference toxicant (chapter 5).

2.1.3.2 Facilities and apparatus

Organisms must be cultured in a temperature-controlled facility. Equipment used to control temperature may include an incubator, recirculating or flow-through water baths, or constant temperature room, but must be able to maintain temperature within the specified range (section 2.1.3.5). The culture area must be isolated from any testing areas or those areas used for the storage or preparation of samples, to avoid contamination from those areas. Culture areas must also be designed and constructed to avoid contamination from other sources. Compressed air delivered to the culture chambers should be uncontaminated, filtered as appropriate to be free of oil and fumes.

All items, chambers, and equipment used in culturing or that may come into contact with the organisms and the culture water must be clean, rinsed, and made of non-toxic materials (e.g., glass, stainless steel, Teflon® or high-density polyethylene (HDPE))[12].

2.1.3.3 Lighting

Overhead full-spectrum lights (e.g., fluorescent or equivalent) should illuminate the cultures and a photoperiod of 16 hours light and 8 hours dark should be maintained. The light intensity at the surface of the water in the cultures should be between 100 and 1000 lux.

2.1.3.4 Culture water

Sources of culture water may include natural waters (e.g., dechlorinated municipal tap water, uncontaminated surface water or groundwater), or reconstituted water[13]. Natural

[12] Glass containers are useful for most COPCs except perfluorinated compounds which tend to plate out on glass. Stainless steel is an option for all COPCs except metals. Teflon® containers can be used for all COPCs except styrenes or perfluorinated compounds. HDPE containers should not be used if the COPCs are organics such as polychlorinated biphenyls (PCBs), dioxin-like PCBs, pesticides or polycyclic aromatic hydrocarbons (PAHs).

[13] It should be noted that this method was developed using dechlorinated Toronto tap water and was not assessed using reconstituted water. Refer to OECD guideline 225 Annex 2 for a recipe and for reconstituted water successfully used in culturing and testing with *L. variegatus* and Annex 3 for characteristics of an acceptable dilution water (OECD 2007). Additional references are available for

water may also be diluted with high quality distilled or deionized water. Acceptable water must allow the satisfactory survival, growth, and overall health of the test species (section 2.1.3.11).

Certain site-specific investigations may require the use of water collected from the same location as the sediment, in which case the water should be filtered through a fine mesh net (e.g., 30 micrometre (µm) EC 2013). Water potentially containing pathogens may be sterilized using a UV sterilizer or passed through a filter with a pore size of 0.45 µm or less. Acclimation of test organisms to the test water or to changes in culture water from sediment is generally not required, although this may be determined on a project-specific basis.

The quality of water in culture chambers should be monitored and recorded routinely. Temperature should be monitored daily and water quality (e.g., dissolved oxygen, pH, conductivity (and ammonia for static conditions)) from at least one representative culture chamber on a weekly basis.

Culture water should be analyzed for hardness, alkalinity, nitrates, suspended solids, total dissolved gases, metals, pesticides, and any other COPCs, as frequently as necessary (at least once per year but quarterly testing is recommended) to document water quality[14].

Water in the culture chambers should be renewed routinely to maintain water quality (section 1.3.2). Water flow in cultures of *L. variegatus* and fathead minnows should be continuous via a flow-through system or recirculating pump.

2.1.3.5 Temperature

The temperature of the water in the culture chambers should be 23 ± 2°C, as a daily average, with an instantaneous temperature of 23 ± 3°C.

2.1.3.6 Dissolved oxygen, pH and Conductivity

It is recommended that dissolved oxygen, pH and conductivity in water from a representative culture vessel be monitored at least monthly and preferably on a weekly basis. Water used in cultures should be well aerated prior to use. Cultures should be aerated gently using filtered, oil-free compressed air delivered through disposable airline tubing and pipettes (glass or plastic) or air stones. It is recommended that dissolved oxygen be maintained at 80 – 100% saturation for all three species. Culture water pH should be between 6.5 – 8.5 and conductivity should be >100 microSiemens per centimeter (µS/cm) (suggestive of > 25 mg/L total hardness).

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reconstituted waters used with other invertebrate species (Borgmann 1996; Smith et al. 1997, USEPA 2000b).

[14] ASTM (2010) section 3.3 provides guidance on acceptable background contaminant concentrations in culture water and USEPA (2000b) recommends that water should be uniform in quality (monthly ranges of hardness, alkalinity and specific conductance <10% of their respective averages and the monthly range of pH is <0.4).

2.1.3.7 Culturing substrate

Shredded brown paper towel soaked in culture water is recommended as a culturing substrate for *L. variegatus*. This substrate should evenly cover the bottom on the culture chamber (a minimum of 1 cm and maximum of 5 cm depth is recommended) and should be renewed as it breaks down. Density of *L. variegatus* in the tank will determine how frequently paper towel additions will be required. In a flow-through system, the flow should be at a rate that does not cause disturbance of the substrate. *L. variegatus* are tolerant of periodic "fluffing" of the substrate which is helpful to prevent accumulation of food debris and reduce risk of anoxia.

The substrate for *Hexagenia* spp. Should be field-collected sediment from an area that is relatively uncontaminated[15] and known or demonstrated to support mayfly populations. It is recommended, if possible, to collect sediment from a location close to where eggs are obtained. The sediment should be fine textured (i.e., predominantly silt/clay) to enable nymphs to construct and maintain burrows. Attempts have been made to develop and use formulated sediment, which can be standardized across laboratories; however, nymph survival and growth are much better in natural sediments (Hanes et al. 1990 and personal communication J. Ciborowski). Procedures for collecting sediment from field locations are outlined in Jaagumagi and Persaud (1993) or EC (1994).

No substrate is provided for fathead minnows as they are reared from fry to juveniles.

All substrate used for culturing should be analyzed for the COPCs to be evaluated in bioaccumulation tests.

2.1.3.8 Food and feeding

Various food types and feeding regimes have been successfully used in the culturing of these test species and are left to the discretion and experience of laboratory personnel (some recommendations are provided below in section 2.1.3.10). All food types used for culturing and bioaccumulation testing should be analyzed for the COPCs to be evaluated in bioaccumulation tests and it may be of interest to assess each new batch of food to ensure continuing food quality.

2.1.3.9 Whole-body concentration in culture organisms

As an alternative or in addition to quantifying COPCs in culture food and substrate, measuring COPC concentration in culture organisms provides an assessment of background concentration against which test sediment exposed organism COPC concentration should be corrected. Organism concentration incorporates all potential exposure routes and accumulation and should be assessed at least on a yearly basis.

2.1.3.10 Handling organisms

Organisms should be handled as little as possible, but when necessary should be done gently, carefully, and as quickly as possible. Organisms that are dropped, injured, or touch dry surfaces must not be used in testing and should be discarded. Groups of *L. variegatus* may be scooped from cultures with a fine-mesh net and substrate rinsed off

[15] Refer to section 2.2.5 footnote 22 for guidance.

with culture water. They can be transferred between containers with a glass or plastic pipette that has a wide and polished end. Organisms should be released below the water surface. *Hexagenia* spp. nymphs may be removed from the cultures using a net or sieve of an appropriate mesh size, and substrate rinsed off with culture water. Small nymphs can be transferred between containers with a glass or plastic pipette that has a wide and polished end, while larger nymphs may be gently transferred using feather tip forceps. Juvenile fish can be transferred between containers using a net. Organisms should be transferred using items that are clean or designated for culture use to prevent contamination of cultures.

2.1.3.11 Establishing and maintaining cultures

Lumbriculus variegatus

Lumbriculus variegatus should be cultured under flow-through conditions in aquaria with shredded brown paper towel as a substrate (based on USEPA 2000b)[16]. Cultures consist of adults of varying sizes that typically reproduce through asexual fragmentation. Trout starter feed may be provided as food in a slurry[17]. A suggested feeding rate is 6 g/aquaria (e.g., 45 L), three times a week. There are no requirements of worm size for initiating a bioaccumulation test and groups of worms are removed from the culture as needed with no synchronization required. Snails should be added to the *L. variegatus* cultures. The snails[18] serve to keep tanks clean, break down the substrate, and keep fungus and other microorganisms under control. Snails have to be thinned occasionally, however, so as not to compete with the worms.

Hexagenia spp.

Mayflies are difficult to continuously culture so eggs are collected in the field from emergent adults, stored, and then hatched in the laboratory when needed (based on Hanes et al. 1990; Bedard et al. 1992). Eggs should be cooled in a stepwise fashion (e.g., 4°C every 4 d; Friesen 1981) and stored in well-oxygenated culture water in sealed containers at 7 ± 2°C. The recommended storage time is ≤ 12 months, as hatching success can become more variable with increased length of storage (personal communication J. Ciborowski).

To initiate a culture, suitable culture sediment should be autoclaved before being distributed to culture chambers (e.g., ~1 L of sediment in a 7-L aquaria). Sufficient sediment should be added in a uniform layer to each chamber to a minimum depth of 2 cm. Sediment should be allowed to oxidize for a few days and stirred occasionally. Food may also be mixed into the sediment at this time (see below). Culture water is then

[16] Suggested flow rate ranges from 50-150 mL/15 sec. Flow too high will decrease the availability of fine food particles in the substrate and too low can lead to fouling and decreased oxygen concentrations near the substrate.

[17] The effect of food type on the sensitivity of organisms is currently under investigation by the OMOE.

[18] *Planorbella pilsbryi* snails have been used with success to keep *L. variegatus* culture tanks in balance.

added to the chamber and gently aerated with an air stone for at least 24 h prior to the addition of organisms.

To hatch nymphs, eggs should be transferred into Petri dishes (e.g., ~200-300/dish) containing new, well-oxygenated culture water that is 7 ± 2°C. Petri dishes are then allowed to warm to 23 ± 2°C. Petri dishes should be checked daily for signs of hatching[19] or contamination. Newly hatched nymphs can be counted into small containers with culture water and then transferred to culture chambers (e.g., ~600/7-L aquaria). Air stones should be briefly removed from the water to allow organisms to swim to the sediment surface.

Nymphs may be fed a diet of ground cereal grass media or alfalfa powder and fish food flakes[20] Food can be prepared in a slurry, at a concentration of ~63 mg (dry weight)/ml of water. Guidelines for feeding rates based on this concentration are as follows.

Feeding rates for ~600 nymphs/ 7-L aquaria

5 ml addition to sediment after autoclaving

After addition of organisms:
5 ml per week in the first 2 weeks,
10 ml per week in weeks 3 and 4,
10 ml 2 times per week thereafter.

The amount of food should be increased over time as the nymphs grow. Nymphs weighing 20-30 mg (average wet weight) are used in bioaccumulation tests. It takes at least 3 months for nymphs to reach this size, depending on organism density and feeding rates, which may be adjusted as necessary to provide appropriate sized organisms in more or less time. It should be noted that within culture tank size variability can be quite high so the number available at testing size at a point in time will be much less than the number of organisms in the tank.

Fathead minnows

Fathead minnows are reared under flow-through conditions in large aquaria and fed a diet of frozen brine shrimp *ad libitum* on a daily basis (based on USEPA 1987; Bedard et al. 1992). Over time, a range in fish size can develop despite similar fish age. Fish should be sorted regularly into different size classes at least one day prior to test initiation. Juvenile fathead minnows weighing 250-400 mg (average wet weight) are used in testing. It takes from 3 to 6 months for fish to reach this size from eggs. Fish that show signs of disease, stress, or sexual differentiation (e.g., tubercules, ovipositors, colour change) must not be used in tests (see USEPA 1987 for further details).

[19] Depending on the batch of eggs and storage time, hatching may occur in as soon as 6 days or may take a few weeks.

[20] *Hexagenia* spp. food is made up of a 3 to 2 (w/w) ratio of cereal grass media and fish food flakes (based on Bedard et al. 1992). This ratio may be adjusted as required. The cereal grass media should be ground and passed through a 250 µm sieve to ensure that material is available to the nymphs (>250 µm material is reground or discarded). The fish food flakes should be coarsely ground to 1-2 mm flakes.

2.1.3.12 Health criteria

A method of assessing the health of organisms is to identify and monitor any number of 'culture health' parameters. This often includes monitoring mortality and looking for abnormal organism size, shape, colour, behaviour, feeding, and presence of disease or stress. Setting culture health criteria is a means of maintaining quality control in a laboratory culture.

It is difficult to establish and monitor culture health criteria for *L. variegatus* and *Hexagenia* spp. due to the large number of organisms in a culture chamber and the inability to count or observe (in the case of *Hexagenia* spp.) organisms without disturbance. Quantitative health criteria (e.g., % mortality), therefore have not been established. Culture health should be assessed using representative organisms removed from the culture and maintained, in culture water under static conditions at culture temperature for two days prior to testing. Organisms must not be used in testing if high incidences of mortality (>10%) are observed in the replicate vessel. It should be noted that due to their burrowing nature, *Hexagenia spp.* in the replicate vessel should be provided with an inert substrate (e.g. nitex mesh, silicone or fire-polished glass tubes) and maintained in the dark.

Qualitative observations may also be used to assess the suitability of cultures. Organisms that do not respond to gentle prodding must not be used in testing or for initiating new cultures. Indications of low dissolved oxygen in a culture chamber include the presence of *Hexagenia* spp. at the water surface. Worms also appear white when temperature has dropped rapidly. An assessment of doubling time in worms has been suggested as measure of culture health (USEPA 2000b)[21]. It has been suggested that doubling time is roughly 10-14 days (USEPA 2000b, Egeler et al. 2005). It is recommended that doubling time be determined by weighing a subset of individuals (e.g. 1 g) with a standardized amount of substrate and maintaining under culture conditions for a set number of days before re-weighing the worms to determine the attained biomass. Attained biomass over the set number of days should then be control charted to track culture health[21].

No quantitative health criteria have been established for juvenile fathead minnows. Organisms must not be used in testing if high incidences of mortality (>10% in the two days prior to testing), abnormalities, disease, or stress are observed. However, the treatment, recovery, and later use of organisms may be possible at the discretion and experience of laboratory personnel, provided a reference toxicant test is also conducted with the batch of organisms.

In addition to culture health criteria, a method to assess and track the sensitivity of a culture is to routinely perform reference toxicant tests. Ideally, a reference toxicant test should be performed in conjunction with a toxicity or bioaccumulation test with sediment. If a laboratory conducts sediment tests routinely, a reference toxicant test

[21] In-house method development is being undertaken at the OMOE. At time of publication, biomass doubling time for 1g *L. variegatus* in 1g paper towel was 11.6 days (±4, n=8). Preliminary experience indicates that time to doubling as determined by counting numbers of individuals (assessed both in sediment and water) is extremely variable, ranging from 1 to >61 days.

must be conducted once each month. If the organisms were purchased then a reference toxicant test must be conducted on each batch of organisms. The results of the most recent test should be control charted to assess and confirm culture health. Procedures for conducting tests with a reference toxicant for each species are outlined in Chapter 5.

Routine measurement of lipid content might provide useful information on the health of culture organisms (ASTM 2010, USEPA 2000b). This information is typically obtained through samples of pre-exposure organisms on the day a bioaccumulation test is initiated and can be plotted in a control chart for each species (using the same process as for results of reference toxicant tests, refer to Appendix D).

Table 1. Summary of required and recommended conditions and procedures for culturing *Lumbriculus variegatus*, *Hexagenia* spp., and fathead minnows.

Parameter	Required and Recommended Conditions
Source of organisms	- existing government, private, field collected or commercial culture; all organisms from the same source; species identification confirmed
Acclimation	- gradually (≤ 2°C/ day) for temperature or water chemistry differences upon arrival
Water source	- uncontaminated surface water, groundwater, dechlorinated tap water, or reconstituted water
Water quality	- temperature monitored daily, dissolved oxygen at least once a week
Temperature	- 23 ± 2°C daily average, ± 3°C instantaneous
Aeration/oxygen	- aerate gently; maintain dissolved oxygen at 80-100% saturation for fish, 60-100% saturation for benthic invertebrates
Lighting	- overhead full spectrum fluorescent, 100 -1000 lux at water surface; photoperiod of 16 h light:8 h dark
Substrate	- shredded brown paper towel for *L. variegatus*; uncontaminated sediment that supports *Hexagenia* spp. survival and growth; none required for fish
Feeding	- various types, quantities, and rates allowed
Age/life stage for bioaccumulation test	- adult *L. variegatus*; 20-30 mg (average) *Hexagenia* spp. nymphs; 250-400 mg (average) (~3-6 month old) juvenile fathead minnows
Health criteria	- do not use invertebrates that do not respond to gentle prodding; do not use batch of organisms that have >10% mortality in the 2 days prior to testing or show signs of disease, stress or abnormal behaviour.

2.2 Test System

2.2.1 Facilities and apparatus

Tests may be performed in a water bath, environmental chamber, or other facility having acceptable control of temperature and lighting. The test facility must maintain the temperature of all sediment and water in test chambers at a daily average of 23 ± 2°C.

All materials that may come into contact with the water, organisms, or test chambers within the facility must be nontoxic (section 2.1.3.2). Compressed air delivered to the

test chambers should be filtered as appropriate to be free of oil and fumes. All equipment and materials that might come into contact with sediment, water, and test chambers should be chosen to minimize the sorption of chemicals. Borosilicate glass, nylon, high-density polyethylene, polycarbonate, fluorocarbon plastics, and stainless steel (type 316) should be used to minimize chemical sorption and leaching.

Since overlying water in each test chamber is aerated in the test, a supply of disposable glass pipettes and aquarium supply airline tubing is required. Stainless steel or plastic gang valves are recommended for regulating airflow.

The test facility must have the basic instruments required to monitor water quality (e.g., temperature, dissolved oxygen, pH, conductivity, ammonia). In addition, the laboratory should be equipped to facilitate the prompt and accurate measurement of hardness, alkalinity, and (where municipal tap water is used as culture/test water) residual chlorine.

All test chambers, equipment, and supplies that might come into contact with sediment or test water must be cleaned and rinsed with test water, deionized water, or distilled water before use. All non-disposable materials must be washed after use.

2.2.2 Lighting

All test chambers should receive full spectrum (e.g., fluorescent or equivalent) overhead lighting. The light intensity at the surface of the water in the cultures should be between 500 and 1000 lux and should be as uniform as possible across test chambers. A photoperiod of 16 hours light and 8 hours dark should be maintained.

2.2.3 Test chambers

The size of test chamber to use is dependent on the total volume of sediment and overlying water used in the toxicity-screening or bioaccumulation test. It is recommended to use chambers that have fairly consistent proportions (e.g., height ~1.5 times the width), are easy to obtain, made of non-reactive material (preferably glass), and are able to be properly covered. Glass jars or beakers with a capacity of 500-1600 ml are recommended for toxicity-screening tests and 2-4 L glass jars are recommended for bioaccumulation tests. All test chambers must be cleaned before and after use, and rinsed with test water before use. Loose covers for test chambers are recommended to minimize evaporation of overlying water, minimize contamination, assist with holding pipettes for aeration, and prevent the escape of fish. Suitable covers include clean watch glasses, or glass or plastic lids.

2.2.4 Test and control/dilution water

The test water (i.e., overlying the sediment) and control/dilution water (i.e., used in water-only reference toxicant test) may be uncontaminated freshwater or reconstituted water (section 2.1.3.4). This is typically the water used in culturing organisms, but other sources may be used for site-specific investigations. The quality of the test or control/dilution water must be shown to support sufficient survival and growth of organisms before use in any test. Water must be adjusted to the test temperature (23 ± 2°C) before use. Dissolved oxygen content should be between 90-100% saturation and water may be aerated vigorously to achieve this prior to use.

2.2.5 Negative control sediment

Each toxicity-screening or bioaccumulation test must include an experimental control with a test chamber containing negative control sediment. A negative control sediment is relatively uncontaminated[22] sediment that has been shown to support good survival, growth, and normal behavior of organisms. It can be the sediment used for culturing purposes, which is recommended for *Hexagenia* spp. Different negative control sediments may be used for different test species due to preferences of physical characteristics, but organism performance must be evaluated in the sediment prior to its use in a definitive test. The use of a negative control provides a measure of test acceptability, is an indication of typical response and organism health, and is a basis for the interpretation of test data.

Negative control sediment may be collected from the field or formulated in the laboratory. Attempts have been made to develop and use formulated sediment, which can be standardized across laboratories[23]; however, growth of benthic organisms is often lower in formulated sediment than in natural sediments (Hanes et al. 1990; Kemble et al. 1999). Physicochemical characterization of negative control sediment must include particle size, density, percent moisture, percent total organic carbon, and the COPCs evaluated in bioaccumulation tests.

2.2.6 Culture organisms

Whole-body concentrations in culture organisms should be below the target detection limits in Table 2-6 in MacDonald et al. (2008).

2.3 Project Planning

Note: This method requires large volumes of sediment, numbers of organisms, and sufficient time to raise organisms to test size or obtain from a supplier. It is extremely important that decisions regarding scheduling and the project plan be made well in advance.

This method requires that prior to conducting a test the following information is obtained and/or determined:

- the objectives of the study,
- the contaminant(s) of potential concern,
- details on the history of the site including previous assessments of toxicity and/or bioaccumulation (including expected variability in sediment COPCs if known),
- collection technique and identification of a reference sediment (defined in section 3.3.3),
- the number of sampling stations,
- whether samples are to be tested as field replicates or as composites,

[22] ASTM recommends that values within or near the 5th percentile of concentrations reported from 200 sediments near coastal sites within the United States (ASTM 2010). MOE (2008a), Marvin *et al.* (2004) and Mudroch *et al.* (1988) provide surface and/or background concentrations of contaminants in the Great Lakes.

[23] Refer to USEPA (2000) for details on the use and creation of formulated sediment. Refer also to Ciborowski *et al.* (1991).

- number of replicates required (refer to section 1.3.1)
- sampling date and approximate date of test initiation
- the number of laboratory replicates requested (per field replicate).

It is particularly important to establish the analytes to be measured as early as possible, as this determines the amount of tissue needed for analytical methods (see section 2.4.1) and the number of organisms required for testing. Decisions regarding the choice or combination of test species and replicate tissue samples should also be made based on the objectives of the study and historical data and power analysis[24]. Decisions regarding the project plan will also determine the volume of sediment that needs to be collected (section 2.4.2). Good project planning will ensure the best use of resources (sediment, organisms, analytical) and generation of the best possible data.

2.4 Sample Requirements

2.4.1 Organism tissue

The sample requirements of various analytical methods depend on the matrix, analyte, and limit of detection. In some cases microanalyses may allow for lower biomass requirements. Confirm with the laboratory what biomass they require and understand the detection limits and uncertainty associated with the chosen analytical method. Note that it is of interest to err on the side of too much tissue to prevent undesirable increase in uncertainty should there be mortality or difficulty in retrieving organisms. This is especially important for *L. variegatus,* which can be problematic to retrieve. Lipid content must always be measured when organic contaminants are of concern. Refer to table 2-6 in MacDonald et al. (2008) for target detection limits in fish and invertebrate tissue for various COPCs.

2.4.2 Sediment collection and transport

This method uses a loading density of organisms that is standardized to a particular proportion of organic carbon in the sediment. Therefore, the volume of sediment used in a test will be different for each sample because of variations in physicochemical properties (i.e., moisture, bulk density, organic carbon). Table 2 provides a conservative estimate of the volume of sediment required for the different components of this method.

Sediment should be collected from field locations following the procedures outlined in Jaagumagi and Persaud (1993) or EC (1994)[25]. Samples should be placed in food-grade polyethylene bags, sealed with minimal airspace, inside separate opaque sample containers that are sealed and well labeled. Sample and storage containers must be new or thoroughly cleaned containers that are made of non-toxic, relatively inert material. Samples should be kept cool and in darkness during transport and must not be allowed to dry or freeze.

[24] Refer to section 12 ASTM (2010) for guidance on experimental design and minimum number of replicates.

[25] Additional guidance is provided in ASTM (2010) and USEPA (2000b).

Minimum requirements for physicochemical characterization of test and reference sediments include total organic carbon and COPC concentration.

Table 2. Volume of sediment required for different components of a bioaccumulation test

Component of test		**Volume of sediment (L)**	
Physicochemical characterization		1.5	
Toxicity Screening[a]		0.5 per species	
Bioaccumulation (volume/replicate)[c]	no. species	metals	organics, one per contaminant group[b]
	1	0.5 / rep	1.5 / rep
	2	1.0 / rep	3.0 / rep
	3	1.5 / rep	4.5 / rep

[a] This is not the volume required for routine sediment toxicity tests, which is ~3 L. Toxicity-screening test details provided in Chapter 3.
[b] DL-PCB/PCDD/F requires a separate sample from PAH/PCB/OC, therefore double the volume of sediment if compounds from both groups are of concern.
[c] Number of replicates dependent on experimental design

2.4.3 Sample handling: Personal protective equipment and procedures

Sediments tested under this protocol are often collected from areas with high levels of contamination and may contain a mixture of hazardous substances. It is important to use personal protective equipment (PPE) and follow procedures (outlined below) to reduce the risk of exposure of staff working with or in proximity to the sample. These procedures are also necessary to prevent the contamination of cultures, control and test exposures, labware, and work areas.

- Contact with skin should be avoided by always wearing PPE (i.e., gloves, labcoat/coveralls/apron, safety goggles, and shoes); these items should be restricted to the testing laboratory and replaced or cleaned regularly.
- A respirator or fume hood can be used when working with samples suspected to contain volatile or particularly high concentrations of organic compounds; tests may be conducted in a fume hood as long as test conditions (temperature, lighting, etc.) can be maintained.
- All items used in sample preparation and testing must be made of relatively inert material (i.e., glass, stainless steel, Teflon®, high-density polyethylene, as appropriate).
- It is advisable to designate labware (e.g., trays, sieves, pipettes, forceps) for use with cultures and control exposures only.
- Separate waste containers should be designated for each sample for cleaning of instruments and sediment disposal. Containers must be labeled, lined, and sealed.
- Test setup and takedown should be initiated with control exposures.
- All equipment, labware, and the work area must be properly cleaned promptly after use; items that cannot be adequately cleaned should be disposed of.

2.4.4 Reception, storage, and holding time

Once received in the laboratory, all containers should be opened to inspect the integrity of the sample.[26] If a sample appears to have dried (evidence of hardened surface and cracking) or frozen, testing should not be performed and the customer should be contacted immediately. Prior to and between uses in testing, all samples should be stored in a sealed container with minimal headspace. It is required to line containers with a food-grade polyethylene bag or use a new container. It is much easier to maintain minimal airspace over time when samples are in a food-grade polyethylene bag that can be closed. Samples must be stored in the dark, at 4 ± 2ºC.

It is highly recommended to test field collected whole-sediment samples as soon as possible after collection and all attempts possible should be made to begin testing within eight weeks (ASTM 2010). If the COPCs in the sediment are relatively stable compounds and recalcitrant (e.g., high molecular weight compounds such as PCBs), long-term storage may be undertaken at the discretion of the researcher (ASTM 2010; DeFoe and Ankley 1998). Storage of the samples must follow the directions outlined above and deviation from the eight week storage time must be reported in the final report. It is recommended that supporting literature on COPC stability be provided.

2.5 Sources of Error

2.5.1 Interferences

Interferences to organism survival and performance in a bioaccumulation test include sediment characteristics such as:

- lethal concentrations of contaminants;
- biological oxygen demand (BOD), production/release of ammonia or hydrogen sulfide; stress from other water quality variables (section 1.3.2) and
- physical characteristics (e.g., particle size, density) that affect normal invertebrate burrowing and feeding behaviour.

It is recommended to screen all sediment samples for toxicity and suitability prior to use in a bioaccumulation test (see section 3.2). Inter-laboratory testing with *Hexagenia* spp. determined that arsenic bioaccumulation from contaminated sediments was significantly higher in replicates with poor survival (Watson-Leung et al. 2016). It was hypothesized that reduced bioturbation in replicates with low numbers of surviving *Hexagenia* spp. altered the speciation and bioavailability of arsenic. This laboratory's failure to meet the control survival criterion however could also indicate that these organisms were unhealthy or stressed and their detoxification mechanisms were compromised. For this reason it is suggested that control survival must meet acceptability to ensure reliable bioaccumulation results (Watson-Leung et al. 2016).

Interferences to the measurement of different analytes in the tissue samples are specific to individual analytical methods and the appropriate methods should be referred to when selecting labware and other items used in the testing protocol.

[26] Sediment may contain organisms accidentally collected during sampling. The decomposition of these organisms might cause fouling and a buildup of ammonia in the sediment. This can make the sample unsuitable for use, in which case it should be discarded at the discretion of the researcher.

2.5.2 Uncertainty

It is important to identify potential sources of error within a laboratory and to estimate the uncertainty associated with these errors. Sources of uncertainty associated with standard toxicity tests and methods to calculate their relative contribution to that of the test endpoint are outlined in Appendix 4 of (Canadian Association for Environmental Analytical Laboratories 2008).

Chapter 3: Bioaccumulation Testing

3.1 Sample Preparation

3.1.1 Sediment homogenization and description

All sediment (per field replicate or sampling station) collected for testing must [27] be thoroughly homogenized with either a stainless steel spoon or a mechanical mixer (e.g., paint or cement mixer), until visually homogenous. Liquid that has separated from the sample during transport or storage must be mixed back into the sample (unless the interstitial water is being analyzed separately or depending on the objective of the study). It is not recommended to sieve sediments; however, rocks, twigs, large biota, and other debris should be removed using forceps or a gloved hand if possible.

If sieving is necessary to remove these objects, then a 1-2 millimetre (mm) sieve may be used. If a sample contains indigenous organisms that might interfere with the test organisms, these may be removed by pressing the sediment through a fine-mesh sieve (e.g., 0.25-0.5 mm) using a minimal amount of liquid. This must be noted and the physicochemical characteristics of the sediment (e.g., particle size distribution, COPCs) should be determined before and after this latter method of sieving. Other methods for the removal of indigenous organisms and more detailed methods of sieving are discussed in EC (1994).

If multiple containers of sediment are collected and are meant to be tested as one sample (i.e., field replicate), they must be composited and homogenized. This can be done using a large batch bucket and an electric mixer. Composited sediment may be redistributed into smaller (preferably lined) containers for ease of use and storage.

A physical description of each sample should be noted, including colour, texture, odour, and presence of debris or indigenous organisms. Sediment must be homogenized (by spoon or mixer) every time a sample container is reopened for use. If the nature of the sediment appears to have changed since it was first described this should be noted and included in the test-specific report.

3.1.2 Sediment characterization

For each sediment sample, at least one sub-sample must be collected for physical and chemical analyses[28]. The analyses required will be project specific, but must include

[27] It may be of interest to evaluate bioaccumulation from intact cores without homogenization. This must be identified as a deviation to the standard method.

[28] ASTM (2010) section 4.1 provides additional guidance on analytical methodology.

particle size characterization, density, percent moisture, percent total organic carbon[29], and COPCs.

Measurements of sediment density and total organic carbon (TOC)[30] are required to calculate the volume of sediment and the loading density of organisms used in a bioaccumulation test (see section 3.3.2). Methods to determine these values in a timely manner are described below (section 3.1.2.1).

3.1.2.1 Method to determine sediment density

The dry density of a sediment sample must be determined because these are used in the calculation of the volume of sediment required in a bioaccumulation test (see section 3.3.2). The wet and dry density of a sediment sample might also be useful in calculations for the dilution of sediment.

1. Homogenize sediment and transfer a known volume of wet sediment into a pre-weighed vessel (n ≥ 3).
2. If wet density is required for dilution calculations or to estimate moisture content, re-weigh vessel with wet sediment.
3. Dry sediment in vessels in an oven until sediment is completely dry.
4. Cool vessels in a desiccator and reweigh.
5. Calculate the wet and dry sediment density of each replicate using the equation below. Calculate the mean and standard deviation of the wet and dry density of the sediment[31].

$$\textit{Wet or dry density (g/ml)} = \frac{\textit{(weight of beaker with wet or dry sediment – weight of beaker)}}{\textit{volume of sediment}}$$

3.2 Toxicity-Screening Tests

3.2.1 Rationale for toxicity-screening test

With each new sediment sample, it is recommended to conduct a test that screens for toxicity to each species selected for bioaccumulation testing, prior to conducting a 28-day bioaccumulation test. This is based on recommendations made in the ASTM (2010) and USEPA (2000b) methods for conducting a bioaccumulation test with *L variegatus*. This ensures that resources such as time, space, and animals are not wasted if the sample is acutely toxic. In addition to assessing acute lethality, this type of test can also indicate whether there are other issues that might be exacerbated in a longer exposure (i.e., water quality degradation, unwillingness or inability to burrow, loss of biomass, and

[29] Anthropogenic sources of carbon (black carbon) can influence bioavailability of hydrophobic organic compounds and it may be beneficial to quantify the proportion of TOC that is black carbon (NRC 2003).

[30] A more rapid analytical alternative, loss on ignition (LOI), is highly correlated with TOC. Refer to Appendix B.

[31] It may be of interest to confirm that a constant dry weight is achieved by repeating the drying and weighing procedure to confirm that the weight achieved is within an acceptable range (e.g. ≤ 0.3 mg) of the previous weight.

lack of feeding). Toxicity-screening tests are designed to be short and use a minimal number of organisms and volume of sediment. General test conditions should reflect those of the bioaccumulation test (e.g., temperature, lighting, and ratio of sediment to overlying water), but may differ with respect to replication and loading density of organisms. Toxicity-screening tests should be conducted as soon as possible after samples are received in the laboratory to allow for testing to be initiated within the eight week testing window. In this way, if the sample(s) are considered unsuitable for use in a bioaccumulation test, changes might be made to the study plan without wasting the sampling effort. Alternatives for unsuitable samples include requesting standard toxicity tests or testing dilutions of the contaminated sediment (e.g., Watson-Leung et al. 2008). Resampling of sediment outside of the toxic area of the site might also be possible within the same field season if this information is known. At the discretion of the researcher, data from routine toxicity tests may be used in lieu of toxicity-screening tests for the same species.

3.2.2 Replication in toxicity-screening test

A minimum of 4 replicate chambers/treatment are recommended in the ASTM (2010) and USEPA (2000b) procedures for a toxicity-screening test. If the sediment has been previously tested and variability is well characterized, use best scientific principles to determine the level of replication necessary in the toxicity-screening test. A negative control exposure should be included for comparison against expected organism performance[32]. If a strong body of data on laboratory performance with the negative control suggests little variability, use discretion to determine how many replicates of negative control should be included.

3.2.3 Acceptability criteria of toxicity-screening test

The performance-based criteria specified for the ASTM (2010) and USEPA (2000b) toxicity-screening test is that the number of oligochaetes should not be significantly reduced in the test sediment relative to the negative control sediment. It is also specified that organisms should burrow into test sediment, since avoidance may decrease bioaccumulation.

To statistically assess whether survival is significantly reduced relative to the negative control, replication of treatments is required. However, dependent on the historical data available for the test and negative control sediments, a decision could be made to balance the risk of false positive or negative results with the benefit of minimized workload and the unnecessary use of resources (organisms and sediment) in the toxicity-screening test. Comparison to historical control or test performance could identify anomalous results that may require further investigation prior to setting up a full bioaccumulation test.

[32] The value of the negative control is to determine the absence of measurable toxicity/bioaccumulation due to basic test conditions (e.g., temperature, health of organisms, effects of handling, control sediment or water). If researcher experience and culture performance is such that there is confidence that unhealthy animals or abnormal behaviour will be easily noted, it may be deemed unnecessary to include a negative control. Use best judgment.

An alternative to statistical comparison is to compare toxicity-screening test results from un-replicated treatments is to use a set of performance-based criteria (see below). These performance criteria were selected to establish whether a test is considered valid; that is whether control organisms have exhibited a typical and acceptable response. If the response in the negative control has met these criteria, then the toxicity of the test sediment can be assessed by comparison to the same performance-based criteria. This should enable a more rapid and definitive decision to be made regarding whether bioaccumulation testing should proceed with a given sediment sample.

Note: If any of the following performance-based criteria are not met in the negative control exposures, the test should be considered invalid and should be repeated and/or prompt an evaluation of culture health. If any of these criteria are not met in the reference (defined in section 3.3.3) or test sediment exposures, the sediments should be considered unsuitable for bioaccumulation testing. Bioaccumulation tests may still be conducted with unaffected test species at the discretion of the researcher.

The test-acceptability criteria[33] for a toxicity-screening test are:

- ≥ 90% survival,
- ≤ 10% loss of biomass (average or total wet weight[34]), and
- No altered behavior or signs of stress (e.g., lack of burrowing, empty guts in nymphs, etc.).

It is recommended to report the results of a toxicity-screening test be included in the final report. If the sample has been deemed unsuitable for assessing bioaccumulation, this information must be communicated to the customer immediately.

3.2.3 Toxicity-screening test conditions and procedures

General test conditions are the same for the toxicity-screening and bioaccumulation tests. Listed below are the specific procedures for conducting a static, 4-day test to screen for toxicity in a sediment sample before it is approved for use in a bioaccumulation test. These test conditions and procedures are based on existing methods[35] and summarized in Table 3.

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[33] The criterion for survival was based on existing OMOE methods for conducting 4-day water-only tests with reference toxicants. A loss of biomass is not expected in a 4-day test and may be an early indication of stress associated with physical or chemical characteristics of the sediment. The criterion for growth was based on data obtained during method development (OMOE, unpublished).

[34] Although the size range chosen for *L. variegatus* is meant to minimize the likelihood of reproduction during the 4-d test, reproduction can occur, in which case total instead of average wet weight should be evaluated since full regeneration of body segments may not occur during the exposure period. Average wet weight should be measured for fathead minnows and *Hexagenia spp.*

[35] In-house assessment of loading densities of 10 organisms in 25, 50 and 100mL of sediment did not appear to influence the results of the 4-day screening tests based on the field collected sediments tested. Volumes of sediment and water and number of organisms per chamber were chosen to maintain consistency with existing OMOE sediment toxicity test methods and ASTM (2010) and USEPA (2000b) screening test recommendations for *L. variegatus*.

3.2.3.1 Toxicity-screening test set up (Day -1)

The day that organisms are exposed to sediment is designated as Day 0. On the day preceding the start of the test (i.e., Day -1) all samples of negative control, reference, and test sediments must be thoroughly homogenized. For the invertebrate species, 100 ml volumes of sediment should be distributed to test chambers, which are recommended to be 700 ml glass jars (~7 cm inner diameter). For fathead minnows, 325 ml volumes of sediment should be distributed to test chambers, which are recommended to be 1800 ml glass jars (~11 cm inner diameter). Replication is not required for the negative control exposures and 1-3 replicates may be used for reference and test sediments. Sediment should be in a uniform layer in the test chamber. Test water should be gently added to each chamber, minimizing the suspension of sediment. The volume of water added to each chamber should achieve a 1:4 ratio (v/v) of sediment to overlying water (i.e., 400 ml or 1300 ml).

Each chamber should be loosely fitted with a lid. A disposable glass pipette may be placed through a hole in the lid and held in place with “elastration rings”. The tip of the pipette should be placed a minimum of 4 cm above the sediment surface. Test chambers should be placed in the test facility (e.g., water bath, environmental chamber) and connected to airlines. Aeration should be adjusted to a constant stream of bubbles to maintain adequate levels of dissolved oxygen (>2.5 mg/L), while limiting turbulence in the water and suspension of sediment. All chambers should be aerated overnight before the addition of test organisms.

3.2.3.2 Toxicity-screening test initiation (Day 0)

All organisms to be used in testing should not be fed the day of the test (Day 0). On Day 0, measurements should be made of the quality of overlying water (i.e., temperature, dissolved oxygen, pH, conductivity, and ammonia; hardness and alkalinity are recommended especially if metals are suspected as a COPC). A sample of overlying water can be removed from each treatment (no more than 10% volume/chamber) for measurement of water quality parameters. This volume should be replaced with test water. Alternatively, water quality parameters can be measured in the test chamber if methods do not disturb the sediment or contaminate the system. If dissolved oxygen is less than 2.5 mg/L aeration can be increased. If concentrations of ammonia in the overlying water are high (e.g., > 0.2 mg/L unionized ammonia), test chambers may be aerated to a maximum of 7 days before beginning the test, at the discretion of the researcher. If pH is outside the range of 6.5-8.5, conductivity is <100 µS/cm or > 1500 µS/cm, hardness is <25 mg/L calcium carbonate (CaCO3)[36], the water quality may adversely affect the health of the organisms (OMOE 1994). This information is useful in the interpretation of the results.

Test organisms used to begin the test should have an average wet weight of 6-8 mg for *L. variegatus*, > 6 mg for *Hexagenia* spp. being careful not to use nymphs that have begun to show signs of late instar development (i.e., darker, thickened wing pads, larger

[36] Note that in-house assessment of sensitivity to low hardness revealed that the 96-hr LC50 of *Hexagenia spp.* and *L. variegatus* is <0 mg/L.

eyes, lightened colour of eyes, usually >40 mg), and 250-400 mg for fathead minnows.[37]

Lumbriculus variegatus and *Hexagenia* spp. should be separated from the culture chambers and placed in a shallow pan with test water. Fathead minnows should be gently netted from culture chambers into a sorting container. Organisms should be handled as little as possible (section 2.1.3.10) and transferred (by pipette or net) into containers filled with test water until there are 10 organisms per container. The average wet weight (ww) of a subsample of organisms (n ≥ 10) should be determined after they have been euthanized and excess water has been removed either through gentle blotting with a clean lint-free towel (e.g. Kimwipe®) or gentle vacuum suction through a paper filter). Invertebrates should be weighed to 0.1 mg and fish to 1 mg using an appropriate balance. It is recommended to randomly choose a sample to re-weigh as a duplicate every 10 to 20 measurements to assess for balance drift or evaporation/adsorption. A general rule of thumb is that duplicates should be no more than 10-20% different, however this is dependent on many variables. It is advisable for laboratories to control chart their duplicate range to incorporate the variability associated with the drying and weighing methodology, equipment, technician, environmental conditions (e.g. humidity) specific to their laboratory. This may help to identify opportunities to reduce variability in weight measurements (see Appendix D). Organisms should be added to the test chambers with minimal addition of water.

3.2.3.3 Toxicity-screening test maintenance

The test must be conducted at a daily mean temperature (of overlying water) of 23 ± 2°C, ± 3°C instantaneous. Test chambers should be illuminated with a 16 h light, 8 h dark photoperiod using overhead full spectrum (e.g., fluorescent or equivalent) lighting. Light intensity adjacent to the surface of the overlying water should be between 500 and 1000 lux and as uniform as possible.

Organisms must not be fed during the test. On a daily basis, temperature should be measured and organism behavior (e.g., lack of burrowing) must be observed and noted. Dead fish must be removed and it is recommended to measure water quality in vessels where there is high incidence of mortality.

3.2.3.4 Toxicity-screening test termination (Day 4)

The test is terminated after 4 days. At this time, water quality parameters should be measured in the overlying water of each treatment (subsample or in test chamber). All of the sediment should be sieved to recover the invertebrate species (~243 µm and 1 mm mesh size for *L. variegatus* and *Hexagenia* spp., respectively). Fathead minnows should be captured in a net while pouring off overlying water. Organisms should be enumerated and placed in containers filled with test water. Organisms that cannot be

[37] The size range specified for *L. variegatus* represents the typical size of these worms; use of larger worms should be avoided since they may split during the test. The size range chosen for *Hexagenia* spp. allows the screening tests to be conducted with whatever size nymphs are available. In this way, feeding of the cultures can be adjusted as necessary depending on whether the organisms are to be used in a bioaccumulation test. The size range for fathead minnows is the same as that for the bioaccumulation test.

found are assumed to have died and decomposed. Dead organisms should be discarded. Organisms should be rinsed to remove any sediment and euthanized immediately. Excess water should be removed from organisms either through gentle blotting with a clean lint-free Kimwipe® or gentle vacuum suction through a paper filter and the total wet weight of organisms from each test chamber should be measured using an appropriate balance. Any changes in the appearance of organisms (e.g., empty guts, discolouration) should also be noted.

3.2.3.5 Toxicity-screening test endpoints and calculations

The percent survival and growth of organisms should be calculated for each treatment. Response in the negative control exposure should be compared to the performance-based criteria (section 3.2.3) to validate the test. The response of organisms from reference or test exposures should also be compared to these criteria to assess the suitability of these sediments for use in a bioaccumulation test. Decisions can then be made as to whether and how to proceed with bioaccumulation testing (section 3.3).

Table 3. Summary of recommended conditions and procedures for toxicity-screening tests.

Parameter	Recommended Condition and Procedure
Test species	- *Lumbriculus variegatus*, *Hexagenia* spp., or fathead minnows
Test duration and type	- 4-day whole-sediment, static test
Test (overlying) water	- uncontaminated surface water, groundwater, dechlorinated tap water, or reconstituted water; 90-100 % DO saturation
Negative control sediment	- tolerable composition for burrowing species (e.g., culture sediment)
Organism test size (average wet weight-ww)	- *L. variegatus* 6-8 mg preferred, *Hexagenia* spp. nymphs 6-40 mg (6-12 mg preferred), fathead minnows 250-400 mg
No. organisms/replicate	- 10
Test chamber	- 700- or 1800-ml glass jars[a] (~7 or 11 cm diameter, respectively)
Sediment volume	- 100 or 325[a] ml (1:4 v/v ratio of sediment to overlying water)
Overlying water volume	- 400 or 1300[a] ml
Replicates	- 1-3
Temperature	- 23 ± 2°C daily average, ± 3°C instantaneous
Lighting	- 16 h light, 8 h dark, 500-1000 lux full spectrum fluorescent
Aeration	- constant stream of bubbles via a Pasteur pipette sufficient to maintain adequate DO levels (>2.5 mg/L) without causing turbulence, a minimum of 4 cm above sediment surface
Feeding	- none
Observations/ measurements	- test initiation and termination: temperature, DO, pH, conductivity, ammonia; hardness and alkalinity recommended especially if metals are the COPC - daily: temperature, organism behaviour
Endpoints	- survival (%), growth (% change in average ww biomass)
Negative control validity criteria[32]	- ≥ 90% survival - biomass ≤ 10% less than initial wet weight – 1 standard deviation (average ww, total ww if worms reproduce) - No altered behavior or signs of stress (e.g., lack of burrowing, empty guts in nymphs, etc.)
Criteria to assess suitability of reference or test sediments for bioaccumulation testing	- ≥ 90% survival - biomass ≤ 10% less than initial wet weight – 1 standard deviation (average ww, total ww if worms reproduce) - No altered behavior or signs of stress (e.g., lack of burrowing, empty guts in nymphs, etc.)
[a] larger test chambers and volumes are for fathead minnows only	

3.3 Bioaccumulation Test

3.3.1 General test conditions

General test conditions are the same for the toxicity-screening and bioaccumulation tests. Listed below are the specific procedures for conducting a static, 28-day bioaccumulation test. These test conditions and procedures are summarized in Table 5.

The loading density of organisms in a bioaccumulation test is standardized to a ~27:1 ratio of sediment TOC to organism dry weight. Therefore, the volume of sediment required in a test will be specific to each sediment sample and species tested. Once the COPCs have been established and the dry density and TOC content of each sample has been determined (section 3.1.2.1), the volume of sediment required for each sample can be calculated (section 3.3.2).

3.3.2 Calculation of sediment volume for a bioaccumulation test

The volume of sediment required in each test is calculated based on the required biomass, sediment-specific density and TOC, and organism-specific wet to dry weight conversion factors.[38] Calculated volumes are rounded up to the closest 50 ml interval to obtain appropriate working volumes and provide slightly more rather than less TOC. If the total volume of sediment and overlying water exceeds the capacity of the test chamber, then sediment, water, and organisms may be split equally between chambers and pooled at the end of the test.

Note: All calculations are conducted on a dry weight basis and then converted to sediment volume based on the sediment density. This is done because it is easier to distribute the sediments to the test vessels by volume than by weight.

Sample calculation (Sediment required/replicate times 3 replicates):

Sediment: Long Point
This sediment has 110 mg TOC/g sediment dw based on analytical results
*Convert **mg** TOC to **g** TOC for future calculations:*
110 mg TOC /g sediment dw ÷ 1000 = 0.11 g TOC /g sediment dw

Species: Lumbriculus variegatus (L.v.)
L.v. is 13% (0.13) dry weight from our laboratory analysis

We require 5 g ww biomass to conduct chemical analysis for organic COCs
Therefor 5 g ww biomass x 0.13 dw/ww = 0.65 g dw biomass

The method requires 27g TOC/g of dw biomass
Therefor 0.65 g dw biomass x 27 g TOC/g dw biomass = 17.55 g TOC

The weight of sediment required to give sufficient TOC is:
17.55 g TOC ÷ 0.11 g TOC/g dw sediment = 159.5 g dw sediment

[38] Determined 'in-house' for each species.

Sediment density is 0.41 g dw/ml based on analytical results
Therefor 159.5 g dw sediment ÷ 0.41 g dw/ml = 389 or ~400 ml sediment (round up)

We require 3 repetitions of each exposure:
3 x 400 ml = 1200 ml of sediment required for this part of the experiment. See Table 2 for other sediment volume requirements.

Note: The wet to dry weight conversion factors for *L. variegatus, Hexagenia* spp. and fathead minnows are 0.13, 0.15 and 0.18 dw/ww, respectively.

3.3.3 Use of negative control and reference sediments

A laboratory or negative control sediment is relatively uncontaminated sediment that has been shown to promote good survival and growth of test organisms under laboratory conditions. Any negative control sediment must be fully characterized for potential COPCs, prior to being considered for use with a bioaccumulation test. The purpose of the negative control exposure is to assess the integrity of the test system and the general health of the organisms. It is not necessary to statistically compare bioaccumulation from test sediments to the negative control sediment, particularly if bioaccumulation of any COPCs from the negative control sediment has been shown to be negligible. Statistical comparisons of survival and growth are also unnecessary as these are not considered sensitive endpoints in this method, but are important for quality control purposes. Although a negative control exposure must be included with every assessment of an environmental sample, replication of the negative control exposures within each experiment is not necessary. This saves both animal and analytical resources. Repeated use of a negative control exposure over time in each experiment can provide a good indication of typical organism performance and whole body concentrations.

The use of reference sediment, in addition to a negative control, may be dependent on the objectives of the study. Reference sediment is typically collected in a similar location as the test sediment and is meant to reflect the physical composition of the test sediment but is removed from the source of contamination. Comparison with reference conditions is often used in toxicity tests when the approach is to permit no further degradation at a particular site or area of concern (ASTM 2010). If statistical comparisons are to be made between exposures to reference and test sediments, replication between treatments should be the same.

3.3.4 Acceptability criteria of bioaccumulation test

The routine use of reference toxicant tests, as well as negative control exposures in toxicity-screening tests, can provide an indication of culture health before organisms are used in a bioaccumulation test. Test species are selected to be relatively resistant to typical contaminants of concern and long-term exposures.

If the performance-based criteria are not met in the negative control exposures, the health of the organisms should be considered compromised, which must prompt an investigation into the functioning of the test system, handling of test organisms, and evaluation of culture health. Once the issue revealed by this investigation is rectified,

the test might should be repeated. The test-acceptability criteria[39] for a bioaccumulation test are:

- mean survival ≥ 80% in *Hexagenia* spp.;
- mean survival ≥ 90% in fathead minnows;
- no net loss in average biomass of nymphs and fish (an increase in biomass is expected)[40]

[39] Survival is based on existing criteria in OMOE methods with *Hexagenia* spp. and *P. promelas*. Criteria for growth were based on the loss of biomass typically observed in tests during method development.

[40] Ability to retrieve *L. variegatus* biomass is highly influenced by sediment texture and organism splitting. Therefore no acceptability criterion is set for *L. variegatus* biomass.

Table 4. Summary of recommended conditions and procedures for bioaccumulation tests.

Parameter	Recommended Condition and Procedure
Test species	- *Lumbriculus variegatus*, *Hexagenia* spp., or fathead minnows
Test duration and type	- 28-day whole-sediment, static test (replacement of evaporated water with RO water)
Test (overlying) water	- uncontaminated surface water, groundwater, dechlorinated tap water, or reconstituted water; 90-100 % DO saturation
Negative control sediment	- tolerable composition for burrowing species (e.g., culture sediment)
Organism test size (average wet weight-ww)	- *L. variegatus* not applicable, *Hexagenia* spp. nymphs 20-30 mg, fathead minnows 250-400 mg
Biomass/replicate	- dependent on COPC(s); confirm with analytical lab
No. organisms/replicate	- sufficient for required biomass
Test chamber	- 2- or 4-L glass jars (height ~1.5 times width)
Organism loading density	- ~27:1 ratio of TOC to organism dry weight
Sediment volume	- based on sediment-specific density and sediment- and organism-specific moisture content, sufficient for required biomass
Overlying water volume	- 4 times sediment volume
Replicates	- min. 3 (field or laboratory)
Temperature	- 23 ± 2°C daily average, ± 3°C instantaneous
Lighting	- 16 h light, 8 h dark, 500-1000 lux full spectrum fluorescent
Aeration	- constant stream of bubbles via a Pasteur pipette sufficient to maintain adequate DO levels without causing turbulence, a minimum of 4 cm above the sediment surface
Feeding	- none for invertebrates, for fathead minnows ~1% body ww of ground fish flakes/day, fed 3 times a week in a slurry
Observations/ measurements	- test initiation, mid-test, and test termination: temperature, DO, pH, conductivity, ammonia, hardness and alkalinity - daily: temperature, abnormal organism behaviour
Endpoints[b]	- bioaccumulation, survival (%), growth (% change in average ww biomass or total ww biomass for *L. variegatus*)
Depuration	- typically none; up to 24 h in water if desired
Test performance-based acceptability criteria	- mean survival ≥ 80% (*Hexagenia*), ≥ 90% (fathead minnows) - no loss in average ww biomass of nymphs and fish[40]

[a] Chamber size is dependent on sediment TOC. Sediments with low TOC require greater volume of sediment and water and therefore larger chambers. [b] Survival and growth are not considered sensitive endpoints, but are necessary for QC purposes and to assist in the interpretation of bioaccumulation

data. Organism survival is expected based on sediment toxicity-screening tests.

These performance-based criteria should also be applied to the reference and test sediments for the interpretation of test results. That is, if performance-based criteria are met, then the sediment is considered to have had no adverse biological affect. If the criteria are not met in organisms exposed to test sediments, the experiment does not need to be repeated and the bioaccumulation data may still be used; however, survival and growth need to be considered in the interpretation of bioaccumulation results (refer to section 4.2.2). There is the potential that the toxicity-screening tests might not have identified adverse responses that were likely to occur in the chronic exposure of the bioaccumulation test.[41]

3.3.5 Conducting a bioaccumulation test

3.3.5.1 Bioaccumulation test set up (Day -1)

The day that organisms are exposed to sediment is designated as Day 0. On the day preceding the start of the test (i.e., Day -1[42]) all samples of negative control, reference, and test sediments must be thoroughly homogenized. The volume of sediment calculated for each sample (section 3.3.2) should be distributed to test chambers, which are recommended to be 2 or 4 L glass jars (height ~1.5 times width). The size of chamber to use is dependent on the total volume of sediment and overlying water and attempts should be made to maintain a ratio of surface area to volume of sediment that is as consistent as possible. A minimum 2 cm depth of sediment is recommended for burrowing species. Replication is not required for the negative control exposures, but a minimum of 3 replicates (field or laboratory) should be used for reference and test sediments.[43] Sediment should be in a uniform layer in the test chamber. Test water should be gently added to each chamber, minimizing the suspension of sediment. The volume of water added to each chamber should achieve a 1:4 ratio (v/v) of sediment to overlying water.

Each chamber should be loosely fitted with a lid. A disposable glass pipette may be placed through a hole in the lid and held in place with “elastration rings”. The tip of the

[41] During method development the incidence of passed screening tests and failed bioaccumulation tests (false negative) were as follows: fathead minnow survival 0% (n=25), fathead minnow growth 6% (n=17), *Hexagenia spp.* survival 36% (n=22) and *Hexagenia spp.* growth 21% (n=19). Since sediments in which screening tests failed were not generally set up for bioaccumulation tests, little information is available on the chance of a false positive screening test result. The bioaccumulation test growth acceptability criterion was met in one of four *Hexagenia spp.* tests in which the screening test failed for the growth criterion.

[42] If elevated ammonia is suspected or if using a spiked sediment, it may be desirable to allow the sediment and overlying water to come to equilibrium for 7 days prior to the addition of test animals. See also section 3.3.5.2.

[43] Analytical requirements can have a strong influence on the number of replicates and the amount of tissue per replicate used in bioaccumulation testing. The number of replicates to use depends on the objectives of the study and for applied studies it is often a balance between statistical power (if necessary) and workload or number of samples/sites that can be tested. The ASTM (2010) and USEPA (2000b) bioaccumulation methods with *L. variegatus* recommend using 5 replicates with 1-5 g of tissue per replicate. Refer to Appendix C for the coefficient of variation calculated with three replicates in the validation of this method.

pipette should be placed a minimum of 4 cm above the sediment surface. Test chambers should be placed in the test facility (e.g., water bath, environmental chamber), connected to airlines. Aeration should be adjusted to a constant stream of bubbles to maintain adequate levels of dissolved oxygen, while limiting turbulence in the water and suspension of sediment. All chambers should be aerated overnight before the addition of test organisms.

Lumbriculus variegatus should be separated from the mass culture and substrate and held in (23 ± 2°C) test water, with aeration, one day prior to test initiation to facilitate easier collection of groups of worms. Fathead minnows should be sorted for appropriate test size at least one day prior to test initiation.

3.3.5.2 Bioaccumulation test initiation (Day 0)

All cultures to be used in testing should not be fed the day of the test (Day 0). On Day 0, measurements should be made of the quality of overlying water (i.e., temperature, dissolved oxygen, pH, conductivity, and ammonia). A sample of overlying water can be removed from each treatment (no more than 10% volume/chamber) for measurement of water quality parameters. This volume should be replaced with test water. Alternatively, water quality parameters can be measured in the test chamber if methods do not disturb the sediment or contaminate the system. If concentrations of ammonia in the overlying water are high (>0.2 mg/L unionized ammonia)[44], test chambers may be aerated to a maximum of 7 days before beginning the test, at the discretion of the researcher[45].

Note: Organisms should always be held or transferred into test water that has been pre-aerated and is within 2-3°C of culturing/testing conditions. Separation, sorting, and distribution of organisms should always be done in as timely a manner as possible to reduce stress associated with crowding and oxygen depletion.

Lumbriculus variegatus: Previously separated organisms should be placed in a shallow pan with test water. Clumps of worms can be gently picked up using feather tip forceps. The tarred weigh boat should contain a small amount of test water to minimize drying of organisms. Masses of organisms should be weighed to 0.01 g using an appropriate balance. Approximately 1.3 times the required amount of tissue should be added to each weigh boat to account for excess water (e.g., 6.5 g for 5 g biomass required).[46] It should be noted that the added water can contribute significantly to the weight measurement. To obtain a more accurate weight excess water can be removed through various methods such as gentle blotting with a clean lint-free Kimwipe® or gentle vacuum suction through a paper filter and organisms can then be transferred to a tarred weight boat. If using this technique be very careful to return the organisms to water as soon as possible. The mass added to each vessel should be recorded on the

[44] 96-hr water-only LC50 values for unionized ammonia toxicity range from 0.4-1.2 mg/L (pH 6.5-8.6) for *L. variegatus* (Schubauer-Berigan et al. 1995).

[45] ASTM (2010), USEPA (2000b) suggest that static-renewal (twice daily replacement of overlying water) is an option in sediment toxicity tests with low water volumes to avoid changes in water quality characteristics. Hardness, alkalinity and ammonia in overlying water should not vary by more than 50% during the test.

[46] As recommended by Brunson et al. (1998) and adopted in ASTM (2010) and USEPA (2000b).

bench sheet. Organisms are then added to test chambers. A subsample of organisms should be collected, euthanized, and frozen for pre-exposure analysis[47]. The burrowing activity of organisms should be observed in the first few hours.

***Hexagenia* spp.**: The nymphs used in a bioaccumulation test should have an average wet weight of 20-30 mg.[48] Nymphs should be sieved from the culture tanks (e.g., using a 1 mm stainless steel sieve) and placed in a shallow pan with test water. Nymphs should be pipetted into holding containers filled with test water to provide the appropriate biomass/container (e.g., 250 nymphs times ~20 mg each ≈ 5 g). An extra holding container should be provided to collect organisms for pre-exposure analysis and weight measurements[49]. Prior to reaching the estimated number of organisms/container, the average wet weight of a subsample (n = 20) of organisms should be determined. Organisms should be weighed to 0.1 mg using an appropriate balance. The mean weight should be used to determine the number of organisms required/test chamber and these (mean weight and number of organisms) should be recorded. Nymphs should be added to holding containers until the correct number has been reached. All holding containers must contain the same number of organisms. The contents of each holding container should be poured through a fine net and the nymphs immediately transferred to each test chamber. A subsample of organisms should be collected, euthanized, and frozen for pre-exposure analysis[47]. The burrowing activity of organisms should be observed in the first few hours.

Fathead minnows: The fathead minnows used in a bioaccumulation test must be juveniles (refer to section 2.1.3.11) and should have an average wet weight of 250-400 mg. Fish should be gently transferred, using a large net, from the culture chambers into a container with test water. Using a smaller net, fish should be gently transferred into containers filled with test water to provide the appropriate biomass/container (e.g., 12 fish x ~400 mg each ≈ 5 g). All containers must contain the same number of organisms. An extra container should be provided to collect organisms for pre-exposure analysis and weight measurements. After euthanizing in carbon dioxide (CO_2) charged water, the average wet weight of a subsample of fish (min. n = 10) should be determined. Organisms should be weighed to 1 mg using an appropriate balance and the mean weight calculated. The number of fish/container should be recorded on a bench sheet. The contents of each container should be poured through a net and the fish immediately transferred to each test chamber. A subsample of organisms should be collected, euthanized, and frozen (<-10°C) for pre-exposure analysis[47].

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[47] Refer to section 2.4.1 for sample size requirements of tissue for each COPC. The same analytes should be measured for pre-exposure (day (d)-0) and post-exposure (d-28) organisms (refer to section 3.3.5.5). Analysis of COPCs should be determined at least once per year on in-house cultured organisms, after a change in food batch and with each batch of purchased organisms for pre-exposure analysis.

[48] Organisms within the 20 to 50 mg size range can be used in bioaccumulation testing with little difference in response due to organism size. However, the use of very large organisms should be limited to reduce the likelihood of emergence or alteration in contaminant uptake during transformation to adult form.

[49] By collecting the organisms for the calculation of average weight during the distribution of organisms to holding vessels, one can ensure that the organisms are truly representative of the distribution of weights among the test chambers.

3.3.5.3 Bioaccumulation test maintenance

Note: ISO 17025 2005 clause 5.3, requires that tests be terminated when results are compromised by a breakdown in environmental conditions during testing (e.g., temperature is outside of range, power outage shutting off lights and aeration).

The clause states “Tests and calibrations shall be stopped when the environmental conditions jeopardize the results of the tests and/or calibrations.”

The test must be conducted at a daily mean temperature (of overlying water) of 23 ± 2°C, ± 3°C instantaneous. Test chambers should be illuminated with a 16 h light, 8 h dark photoperiod using overhead full spectrum (e.g., fluorescent or equivalent) lighting. Light intensity adjacent to the surface of the overlying water should be between 500-1000 lux and as uniform as possible. Water that has evaporated from the test chambers should be replaced with a water low in ions (e.g., reverse osmosis (RO), distilled or deionized (DI) water).

Feeding[50]: Invertebrate species must not be fed during a bioaccumulation test. Fathead minnows are fed a diet of crushed (< 2 mm) fish food flakes at a rate equivalent to ~1% of their wet body weight per day. This food is delivered to each test chamber as a slurry (blended with dechlorinated water and delivered in no more than 3 mL/test chamber) 3 times per week. The amount of food added to each test chamber should be adjusted if fish have been removed due to mortality.

Monitoring: Temperature should be measured daily. It should be ensured that all test chambers are properly aerating. Any abnormal behaviour of organisms should be noted. Dead fish must be noted and removed and it is recommended to assess overlying water quality (especially D.O. and ammonia) in the vessels in which dead fish were found. Midway through the test, water quality parameters should be measured in the overlying water of one replicate from each treatment (subsample or in test chamber).

3.3.5.4 Bioaccumulation test termination (Day 28)

Note: Test termination should be initiated with negative control exposures to prevent contamination and caution should be used to avoid cross-contamination.

The test is terminated after 28 days. At this time, water quality parameter should be measured in the overlying water of one replicate from each treatment (subsample or in test chamber)[51]. The remaining water can be collected for chemical analysis if deemed necessary by the project plan.

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[50] Feeding has been discouraged in bioaccumulation tests as organisms may preferentially ingest the food instead of sediment, limiting the uptake of contaminants (ASTM 2010). Fathead minnows do not actively ingest sediment as a food source and comparative studies show feeding may be required to ensure organism survival and health during 28 day test and percent lipid was dramatically lower in unfed fish exposed to the same sediments which also led to higher body COPC concentrations. Total PCB BSAFs were significantly different (p=0.005, n=8 paired t-test) between fed and unfed fathead minnows exposed for 28-days to field collected sediments.

[51] If time and labour is limited for test termination on day 28, overlying water quality parameters could be measured on day 27 since they have been shown not to vary between day 27 and 28.

If conducting a mass balance assessment, ensure that all overlying water is collected and the fines are allowed to settle.

Using a pan to contain the sample, sediment should be passed through a sieve to recover invertebrates (~243 µm and 1 mm mesh size for *L. variegatus* and *Hexagenia* spp., respectively). Fathead minnows should be captured in a net while collecting/pouring off overlying water. Organisms should be transferred to containers filled with test water, enumerating nymphs and fish while doing so. Oligochaetes should be transferred using a pipette and nymphs may be transferred using fine forceps. Missing individuals are assumed to have died and decomposed during the test. Organisms from any treatments that were split between test chambers should be pooled, although it may be of interest to obtain per split weights prior to pooling the organisms. Since this bioaccumulation method was developed with the intention of using the data for risk assessment, field to lab comparisons and food web modeling it is not standard practice within this method to allow organisms to purge sediment from their guts. Refer to section 3.3.5.5 for more details on gut purging and guidance on how to terminate a test with gut purged organisms.

Organisms should be euthanized soon after recovery to avoid depuration. Organisms should be rinsed to remove any sediment. The total wet weight of organisms in each replicate should be determined to 0.01 g using an appropriate balance. The water from the containers holding *L. variegatus* should be poured off and the worms transferred to a weigh boat with forceps, touching clumps of worms to the side of the beaker to remove excess water **(must not be blotted on a towel)**. Nymphs and fish can be collected in a fine sieve or net and blotted before being transferred to a weigh boat.

3.3.5.5 Gut Purging

Under certain circumstances gut purging may be desired (e.g., metals, kinetics, spiked sediment, research-type investigations). During the development and standardization of this test method, a literature review was conducted. As a result of the outcome of the literature review, the following review of gut purging was published by Van Geest *et al.* (2010c).

> "Contaminants associated with sediment remaining in the guts of organisms potentially leads to artificially high estimates of tissue concentrations. Purging organisms in clean conditions before analysis is a means of reducing or eliminating this bias. However, depuration and metabolism of compounds can occur during purging, leading to an underestimate of tissue concentrations. A number of errors associated with gut sediment and purging are summarized in the ASTM guidance document (Table 4, p. 1101, in ASTM (2000). Purging of L. variegatus in clean sediment has been shown to enhance depuration, possibly leading to the dilution of total body burden with uncontaminated sediment [Kukkonen and Landrum 1994; Kukkonen and Landrum 1995; Leppänen and Kukkonen 2000; Ingersoll *et al.* 2003). Increased depuration in sediments with higher TOC also has been observed in sediment- and soil-ingesting species [Lydy *et al.* 1992; Belfroid and Sijm 1998). Purging appears to have less effect in fish than invertebrates because of the smaller contribution of gut sediment to total body weight (Mac *et al.* 1990).

Organisms were purged in more than 70% of the studies we reviewed, half of which purged for approximately 24 h. Purging times varied from 6 h (You et al. 2007) to approximately 24 h (Harkey et al. 1995; Pickard et al. 2001) or 48 to 72 h (Mac et al 1990; McLeod et al. 2008). Brooke et al. (1996) determined that the inorganic contents of the gut represented approximately 10% of the whole body dry weight in unpurged H. limbata, C. tentans, and L. variegatus, and that these species lost 75, 90, and 100%, respectively, of their gut contents in the first 12 h of water-only purging. Mount et al. (1999) evaluated purging of sediment in L. variegatus and found that only 6 h were required to eliminate more than 98% of gut contents. As a result, a 6- to 8-h purge for L. variegatus is recommended in the ASTM (2000) and USEPA (2000b) methods. Many of the compounds of interest in bioaccumulation studies have log KOW values greater than 5, in which case Mount et al. (1999) predict that 90% accuracy in estimates of tissue concentrations, with little or no bias from gut contents, would be observed in purges as long as 24 h. The selection of a purging period may need to consider the accuracy of estimates and the contaminants being measured. As an alternative to purging, corrections for the contribution of gut contaminants to total body burden can be applied using the mass of gut content and concentration of the contaminant in sediment (suggested by Chapman 1985 and Neumann et al. 1999).

A number of situations that arise when purging may not be necessary or should not be conducted. These include laboratory– field comparisons, or using bioaccumulation data to determine trophic transfer of contaminants. Under environmental conditions, a predator eats the whole prey and is therefore exposed to contaminants associated with gut sediments. Purging is also not recommended with low-molecular-weight compounds, such as PAHs, which may be quickly depurated (ASTM 2010).

In the definitive testing for the assessment of this bioaccumulation method the ratio of concentrations of contaminants of purged to that of unpurged organisms was examined. It was found no one species consistently had the highest ratios across the sediments tested, suggesting that the presence of sediment in the gut did not have a greater contribution in any particular species. Purging of organisms reduced the variability of estimates in *Hexagenia spp.*, but this was not the case for *L. variegatus* or *P. promelas.* Results of this validation study suggest that for the intended use of bioaccumulation data (i.e., risk assessment and food web modeling) there is no advantage to purging organisms since the variability in the estimate of tissue concentrations of organic contaminants is not always reduced."

If the test is to be terminated with purged organisms, the organisms from each replicate should be placed in a chamber with ~1.5 L of test water, or if the COPC(s) is/are metals

then purge in 50 micromole (µmol) Ethylenediaminetetraacetic acid (EDTA) solution[52]. These chambers should be placed in the test facility (e.g., water bath or environmental chamber) with aeration for the desired period of time (up to 24 h[53]). To reduce stress associated with continual swimming, nymphs should be provided with a mesh substrate and overhead lighting turned off or in darkened vessels. Purging of gut contents may be more relevant when metals are of concern. It may be of interest to periodically clean out feces during the purging period to prevent coprophagy. If organisms have been purged this MUST be noted in the final report along with purging time.

3.3.5.6 Sample preparation for chemical analysis

Biota: Organisms should be transferred from each replicate into separate glass vials (foiled lined lids for organics and Teflon® lined lids for metal analysis). Vials should be well-labeled with a sample, organism, and replicate code for the project. When dl-PCBs and polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran (PCDD/F)s are the contaminant of concern small amounts of tissue may be subsampled from each replicate and pooled for lipid analysis (e.g., 1-2 g), since these require separate analyses. All samples should be kept frozen (≤-10°C) until chemical analysis. Pre-exposure[54], negative control, and test samples must be analyzed for the same COPCs and lipid content (for organic compounds).

Overlying water: If day 28 overlying water is collected, suspended solids should be allowed to settle out of water, leaving the sample overnight if necessary. Appropriate sample bottles should be filled and preserved as needed for the contaminants to be measured. Samples should be stored at 4 ± 2°C until chemical analysis.

Sediment: If day-28 sediment is collected, sample should be allowed to settle and the excess water poured off. The sediment should be transferred to the appropriate sampling container and stored at 4 ± 2°C until chemical analysis.

3.3.6 Test endpoints and calculations

The biological endpoints determined in this test are survival, growth, lipid content (where appropriate), whole-body concentration of the COPCs, and a biota-sediment accumulation factor (BSAF) of the COPCs. The following endpoints should be calculated for each treatment:

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[52] Options for compensation of gut contents in the measurement of body burden for sediment ingesting invertebrates include removal of gut contents by dissection, purging of gut contents in clean water or clean sediments or correction of the whole unpurged animal contaminant concentration by the proportion contributed by gut contents (Hare et al. 1988; Chapman et al. 1980). Gut content contribution to total weight ranged from 2.5-22% for *Hexagenia limbata* (Hare et al. 1988; Brooke et al. 2009) and 10% for *l. variegatus* (Brooke et al. 2009).

[53] The ASTM (2010) and USEPA (2000b) bioaccumulation methods recommend purging *L. variegatus* for 6-8 h. A purging period of up to 24 h has been provided as an option in this protocol as this is more feasible for a method broadly applied in a regulatory and monitoring context. If organisms are purged, purging time must be reported.

[54] If the same sediment is used for rearing organisms and as a control (i.e., with *Hexagenia* spp.), pre-exposure organisms may be measured only for lipid content when dl-PCBs or PCDD/Fs are of concern. This is due to the requirement of separate tissue samples for analyses.

- mean (± standard deviation; SD) of percent survival (for *Hexagenia* and fathead minnow only);
- mean (± SD) percent growth (change in average biomass (*Hexagenia* and fathead minnow) or total biomass (*L. variegatus*) from Day 0 mean);
- mean (± SD) percent change in lipid content (from Day 0; when measured);
- mean (± SD) tissue concentration of the COPCs; and
- mean (± SD) BSAF of the COPCs.

Response in the negative control exposure is compared to the performance-based criteria (section 3.3.4) to validate the test. The response of organisms from reference or test exposures should also be compared to these criteria to assess the severity of any adverse biological effects and assist in the interpretation of bioaccumulation results.

3.3.6.1 Whole-body concentrations[55]

Whole-body concentrations are reported on a wet or dry weight basis depending on the analytical method used. In the literature, reporting often varies between studies; however, metals are typically reported on a dry weight (dw) basis and organic compounds on a wet weight (ww) or lipid basis. Regardless of these practices, this information (dw, ww, or lipid basis) must be clearly indicated in the final report. If a wet to dry weight conversion factor (or vice versa) was applied to data this must also be identified.

For comparison between species, whole-body concentrations of organic contaminants are often normalized to lipid content. *If whole-body concentrations are normalized for lipid this must be done before the mean and SD are calculated.*

Whole-body concentrations do not need to be corrected for pre-exposure concentrations unless the COPCs were detected above trace levels in the pre-exposure organisms. If corrections are made this must be reported. Tissue concentrations in pre-exposure organisms should be below the target detection limits in Table 2-6 in MacDonald et al. (2008).

In addition to reporting absolute tissue concentration, a BSAF[1] can also be reported. This factor represents the bioaccumulation of a contaminant relative to the concentration in the sediment. In it's simplest form, typically used for metals and contaminants other than nonionic compounds (Van Geest 2010), it is calculated as;

$$\mathrm{BSAF} = \frac{C_O}{C_S}$$ e.g., mercury or arsenic

where C_O is the concentration of COPC in the organism (nanogram per gram (ng/g) dry weight)[56] and C_S is the concentration in the sediment (ng/g dry weight).

[55] If organisms were purged the organism COPC concentration can be referred to as tissue concentration. It should be noted that without gut purging the measured whole-body COPC concentration may not reflect bioaccumulation by tissues or the fraction of the chemical that was bioavailable to the organism. This method was validated without gut purging organisms although the impact of gut purging was examined (Van Geest *et al.* 2011c). Refer to section 3.3.5.5.

Characteristics such as sediment TOC content and organism lipid content greatly influence the bioavailability and partitioning of nonionic organic contaminants between sediment, (pore) water, and organism. Normalizing sediment and tissue concentrations for these parameters is the accepted practice to reduce variability both within and between species (Van Geest 2010). For most non-ionic organic contaminants, the BSAF[1] is defined (Ankely et al. 1992; Burkhard 2009) as;

$$\text{BSAF} = \frac{C_O / f_\ell}{C_S / f_{SOC}}$$

where C_O is the concentration of COPC in the organism (ng/g wet weight), f_ℓ is the lipid fraction of the organism (g lipid/g wet weight), C_S is the concentration in the sediment (ng/g dry weight *)* and f_{SOC} is the fraction of the sediment as organic carbon (g organic carbon/g dry weight)[57].

The units must be reported to identify whether the ratio has been derived using wet or dry weight concentrations and/or normalized for lipid and TOC content. Based on the typical procedures in this method these ratios are calculated based on one measurement of sediment concentration made during initial preparation and subsampling of the sediment. However, if sediment was sampled from each replicate test chamber, a BSAF could be calculated for each replicate exposure. *The data that is to be reported should be the mean of the BSAFs and not the BSAF of the mean tissue and sediment concentrations*. This allows the SD to be reported instead of the error associated with the mean concentrations to be compounded.

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[56] Tissue is often reported on a wet weight basis and should to be converted to dry weight prior to BSAF calculation for metals. The wet to dry weight conversion factors determined by the MOECC ATU for *L. variegatus, Hexagenia* spp. and *P. promelas* are 0.13, 0.15 and 0.18, respectively.

[57] Note that lipid and TOC are often reported as %. An example of conversions required for BSAF calculations when % or other until values are reported is:
e.g. 0.5% = 5 mg/g = 0.005 g/g = 0.005 f_ℓ or f_{SOC}

Table 5. Summary of recommended conditions and procedures for bioaccumulation tests.

Parameter	Recommended Conditions and Procedures
Test species	- *Lumbriculus variegatus*, *Hexagenia* spp., or fathead minnows
Test duration and type	- 28-day whole-sediment, static test (replacement of evaporated water with RO water)
Test (overlying) water	- uncontaminated surface water, groundwater, dechlorinated tap water, or reconstituted water; 90-100 % DO saturation
Control sediment	- tolerable composition for burrowing species (e.g., culture sediment)
Organism test size (average wet weight-ww)	- *L. variegatus* not applicable, *Hexagenia* spp. nymphs 20-30 mg, fathead minnows 250-400 mg
Biomass/replicate	- 1.5 g ww for metals, 5 g ww for organic compounds
No. organisms/replicate	- sufficient for required biomass, generally 1g metals, 5 g organics[a]
Test chamber	- 2- or 4-L glass jars (height ~1.5 times the width)
Organism loading density	- ~27:1 ratio of TOC to organism dry weight
Sediment volume	- based on sediment-specific density and sediment- and organism-specific moisture content, sufficient for required biomass
Overlying water volume	- 4x sediment volume
Replicates	- min. 3 (field or laboratory)
Temperature	- 23 ± 2°C daily average, ± 3°C instantaneous
Lighting	- 16 h light, 8 h dark, 500-1000 lux full spectrum fluorescent
Aeration	- constant stream of bubbles via a Pasteur pipette sufficient to maintain adequate DO levels without causing turbulence, a minimum of 4 cm above sediment surface
Feeding	- none for invertebrates, for fathead minnows ~1% body ww of ground fish flakes/day, fed 3 times per week in a slurry
Observations/ measurements	- test initiation, mid-test, and test termination: temperature, DO, pH, conductivity, ammonia - daily: temperature, organism behaviour
Endpoints[a]	- bioaccumulation, survival (%), growth (% change in average ww biomass or total ww biomass for *L. variegatus*)
Depuration	- typically none; up to 24 h in water if desired
Test performance-based acceptability criteria	- mean survival ≥ 80% (*Hexagenia* spp.), 90% (fathead minnows) - no loss in average ww biomass of nymphs and fish - no criteria for *L. variegatus*[b]

[a] Refer to Table 13.5 in USEPA 2000b.

[b] Survival and growth are not considered sensitive endpoints, but are necessary for QC purposes and to assist in the interpretation bioaccumulation data. Organism survival is expected based on sediment toxicity-screening tests.

[c] Sediment texture and debris can dramatically affect retrieval success for *L. variegatus.*

Chapter 4: Reporting Guidance

4.1 Reported Endpoints and Interpretation of Data

The interpretation and reporting of data from a bioaccumulation test is based on the study design (see section 2.3). A test-specific report must include all data used for the interpretation of survival and bioaccumulation results, where further interpretation beyond basic reporting is warranted.

4.1.1 Reported endpoints

Survival/biomass must be reported for all treatments, unless the bioaccumulation test was not conducted, in which case survival, growth, and observations in the toxicity-screening test must be reported.

Tissue concentrations of the COPCs must be reported for reference and test treatments. Concentrations in pre-exposure and negative control organisms must be reported but may be assessed on an annual basis for the laboratory culture and negative control exposed organisms. If concentrations in pre-exposure and negative control organisms were detected above trace levels this must be considered in the interpretation of bioaccumulation results from reference and test treatments.

The BSAF of the COPCs must be reported for each test treatment. If the reference sample contains low or very low concentrations of the COPCs, it might not be appropriate to calculate or report a BSAF.[58]

Change in biomass and lipid content of organisms only need to be reported if the metabolic health of organisms appears to have been adversely affected (see section 4.1.2.1) and/or these data are considered in the interpretation of survival and bioaccumulation results.

When data are reported the following must be included when appropriate: mean, standard deviation, and coefficient of variation. Statistical guidance outlined in EC (2005) should be followed where appropriate. Results of the corresponding or most recent reference toxicant test must be reported when applicable. Measurement of uncertainty may be reported at the request of the customer. Supplemental information not pertinent to the interpretation of results, but used in conducting and validating responses in the test may be included in an appendix to the test-specific report (e.g., sediment density, and moisture content, organism growth, lipid content, or change in lipid content). Reporting requirements are further outlined in section 4.2.

4.1.2 Guidance for the interpretation and use of data from bioaccumulation tests

4.1.2.1 Data Analysis

This section will not provide specific details on how to perform data analysis but is written to provide guidance on statistical procedures available and reference documents

[58] This is because BSAFs are not always independent of sediment concentration, as assumed in equilibrium partitioning models, and higher BSAFs sometimes result for sediment with low concentrations of TOC or contaminants (Mc Elroy and Means 1988; Lake et al. 1990).

that will assist in data analysis. It is recommended that a statistician be involved at all stages of a test from study design to data analysis (EC 2005).

The main objective of bioaccumulation testing is to determine the tissue residues of COPCs in exposed aquatic organisms. To perform statistical analysis of these residues, replication of sample exposures is required

It should be understood that lab replicates (replicate exposure vessels from a single composite sample of sediment from the site) provide uncertainty in the method while field replicates (single exposure vessels with replicate sediment samples from the site) provide an estimate of site variability. Mean, standard deviation, coefficient of variation (CV) and number of replicates should always be reported. The CVs for this method with three laboratory replicates is provided in Appendix C. Refer also to table A2.1 in ASTM (2010). It should also be noted that analytical uncertainty plays a part in these and it is recommended that method analytical uncertainty be incorporated into a power analysis when making a decision about replication. Refer to section 16.2.3 in the USEPA (2000b) test method on Hypothesis Testing and Power for more details on how to determine the number of replicates necessary based on the project objectives.

Guidance on statistics analysis and interpretation of results and other considerations of test design can be found in ASTM (2010) section 14 and 15 and Appendix A1, EPA (2000) section 16 and EC (2005).

4.1.2.2 Organism survival, growth and lipid content endpoints

The rationale for the selection of biological endpoints (survival, growth, lipid content) in addition to bioaccumulation has been discussed earlier in this document (section 1.3.3). The performance-based criteria established for the different test organisms (listed in section 3.3.4) are used to validate the test and assess the severity of any adverse biological effects observed in reference and test treatments. In addition, these endpoints can provide information into the uptake, metabolism and depuration activity of the exposed organisms. For example, it is known that reproducing *L. variegatus* cease feeding for two to 7 days (Leppänen and Kukkonen 1997) which may lead to a reduction in exposure to contaminants. Comparison of tissue concentrations between sediments where there is a dramatic difference in growth in test organisms should consider the impact of growth dilution, the dilution of the concentration of contaminant by the increase in tissue volume (Arnot and Gobas 2006), during data interpretation.

It is important to have an understanding of the large number of factors affecting sediment contaminant bioavailability and organism uptake. See Leppänen (1995) for a review on the impact of sediment characteristics and organism feeding behavior on bioaccumulation.

Evidence that the metabolic health of organisms was compromised in a test (i.e., loss of biomass or lipid) might need to be considered in the interpretation of bioaccumulation results. Some loss of lipids is not completely unexpected in a 28-d test, since organisms are moved from culturing conditions in which high quality food is provided on a regular basis to a potentially nutrient limited exposure. The growth of organisms without the same relative increase in lipid will also result in a decrease in lipids as a percentage of the whole body weight. *Therefore, if biomass increases throughout a test it can be*

assumed that the organisms are relatively healthy, even if the percentage of lipid decreases. In instances where lipid content decreases with little or no change in biomass, this suggests that organisms have entered a state of starvation metabolism, in which stored lipid is actively metabolized as a source of energy. Yet the loss of lipid might not be severe enough to result in the loss of total biomass. Stress associated with a state of starvation metabolism might also affect the organisms' sensitivity to contaminants and might contribute to any observed mortality. Sites in which organism metabolic health appears to be impaired may have contaminant concentrated in tissue with loss of lipid or enhanced depuration of contaminant through mobilization of lipids.

4.1.2.3 Tissue concentrations

Comparison of tissue concentrations between negative control and pre-exposure organisms provides an indication of the functioning of the test system and whether contamination has occurred. Tracking the average and standard deviation around tissue concentrations of pre-exposure and/or negative control exposed organisms is recommended. The collection and presentation of tissue concentration data in this manner and knowledge of the typical range in concentrations for pre-exposure and negative control exposed organisms might assist in identifying data that is suspect. Comparison of tissue concentrations in organisms of reference or test treatments to pre-exposure and/or negative control organisms indicates the bioaccumulation of the COPCs. Comparison of tissue residues between reference and test treatments indicates the extent of bioaccumulation of these COPCs relative to assumed background or low exposure areas.

4.1.2.4 Lipid content

For organic contaminants, normalization of tissue concentrations for lipid content is a means by which to reduce variability both within and between species. The lipid content of pre-exposure and negative control organisms has been shown to vary in the three test species, without organism health appearing to be affected.[59] Plotting the lipid content of pre-exposure organisms on a warning chart has been recommended as an additional means to assessing culture health (section 2.1.3.12). The collection and presentation of lipid data in this manner and knowledge of the typical range in lipid content for a test organism might assist in identifying lipid data (from pre-exposure, negative control, reference or test organisms) that are suspect.

Different methodologies for analyzing lipid can result in different lipid concentrations[60]. Therefore, care should be taken and the methods noted when lipid data are provided by another laboratory or comparisons are made to other studies.

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[59] During method development, average (±sd) lipid content of pre-exposure *L. variegatus* (n=9), *Hexagenia spp.* (n=8) and *P. promelas* (n=6) was 1.1 (±0.32), 1.3 (±1.4) and 6.6 (±1.9) %, respectively. Average lipid content after 28-day exposure to negative control sediment was 0.6 (±0.3), 0.8 (±0.6) and 4.6 (±1.2) % for *L. variegatus* (n=10), *Hexagenia spp.* (n=10) and *P. promelas* (n=7), respectively. Refer also to Appendix C for uncertainty around measured lipid content in pre-exposure, control and test organisms during method development.

[60] Refer to section 14.2 in ASTM (2010).

4.1.2.5 Biota-sediment accumulation factors

Biota-sediment accumulation factors are a means by which to assess relative bioaccumulation differences among individual chemicals, between sediment samples, sites, or over time. For example, BSAFs for a certain COPC can be compared among sediment samples within a site, which may differ in contaminant concentration and TOC content. Variability in contaminant bioavailability within a site may come from varying contributions of anthropogenic sources of carbon (e.g. black carbon). It should be understood, however, that BSAFs are not a measure of hazard in that sediments with the highest concentration of contaminants do not always lead to the highest BSAFs. Relatively lower BSAFs sometimes result with sediments that contain very high concentrations of contaminants due to the potential for maximum concentrations that can be accumulated in the organism, altered exposure if organism health is compromised by the degree of contamination or sediment characteristics that reduce the bioavailability of the COPC. Relatively higher BSAFs can also occur in sediments with very low concentrations of contaminants and/or TOC.

BSAFs have also been used as models to predict tissue residues directly from sediment concentrations, reducing the need for field and laboratory studies. However, this approach relies on the assumption that BSAFs are constant among species and sediment. A suggested application of this approach is to determine a site-specific BSAF for a COPC based on laboratory testing of one or more sediment samples from a site, and to use this (mean) BSAF to predict the potential tissue residues of organisms based on other sediment concentrations within the site. This makes use of the spatial data of sediment chemistry that are often collected during site investigations and limits the amount of sediment and number of samples to collect for bioaccumulation testing, as well as laboratory workloads and use of organisms.

BSAFs can be compared to bioaccumulation models based on equilibrium partitioning, which typically predicts that the maximum BSAF for neutral organic compounds is approximately 1.7 (McFarland and Clarke 1986). BSAFs lower than this theoretical maximum might result from chemical disequilibrium, metabolic processes such as biotransformation or elimination, steric hindrances, or other violations of the basic assumptions of the model including independence of sediment concentration. BSAFs greater than this maximum, in the range of 3.5 to 4, were observed in some sediments used in the validation of this method, however this is within the range of analytical variability for the analytes

4.2 Reporting Requirements

Each test-specific report must indicate whether there have been any deviations from the requirements ('*must*' statements) in this method, and the details of these deviations. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the intended use.

Section 4.2.1 provides a list of items that must be included in a test-specific report. Items that must either be included in the test-specific report, provided separately in a

general report, or held on file for a minimum of five years are listed in section 4.2.2. Specific monitoring programs, related test protocols or regulations might require that selected test-specific items listed in section 4.2.2, be included in the test-specific report.

Procedures and conditions common to a series of ongoing tests and consistent with specifications in this document may be referred to by citation or attachment of a general report which outlines standard laboratory practices.

Details on the conduct and findings of the test, which are not conveyed in the test-specific report or general report, must be held on file by the laboratory for a minimum of five years so that the appropriate information are available if an audit of the test is required. Filed information might include:

- a record of the chain of continuity/custody for field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition of sample(s);
- chemical analytical data of the sample(s) not included in the test-specific report;
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicant tests;
- detailed records of the source of organisms, their taxonomic confirmation
- detailed records of all pertinent information on organism culturing and health; and
- information on the calibration of equipment and instruments.

Original data must be signed or initialed, and dated by the laboratory personnel conducting the tests.

4.2.1 Minimum requirements for a test-specific report

The following is a list of items that must be included in a test-specific report.

4.2.1.1 Test substance or material

- a brief description of sample type (e.g., dredged material, reference or contaminated, field-collected sediment, negative control sediment) or coding, as provided to laboratory personnel;
- information on labeling and coding of each sample; and
- date of sample collection; date and time sample(s) received at test facility.

4.2.1.2 Test organism

- species and source of brood stock and test organisms;
- range of size, at start of test (where applicable); and
- any unusual appearance or treatment of organisms before their use in the test.

4.2.1.3 Test facilities

- name and address of test laboratory; and
- name of person(s) performing the test.

4.2.1.4 Test water

- type and source of test water.

4.2.1.5 Test method

- citation of biological test method used (i.e., as per this document);
- design and description if specialized procedure (e.g., sieving or dilution of field-collected test sediment) or modification of standard test method;
- brief description of frequency and type of observations and measurements made during the test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

4.2.1.6 Test conditions and procedures

- design or descriptions if any deviation from or exclusion of any of the procedures or conditions specified in this document;
- number of discrete samples per treatment, number of replicate test chambers per treatment, number and description of treatments in each test including the control(s); test concentrations (if applicable);
- volume of sediment and overlying water used in each test chamber;
- number (or total mass) of organisms per test chamber and treatment;
- feeding regime and ration (where appropriate);
- dates when test was started and ended;
- for each sample – all measurements of sediment TOC and COPCs; and
- for at least one test chamber representing each treatment – measurements of water quality (i.e., temperature, dissolved oxygen, pH, conductivity, and ammonia) made during the tests; data may be reported as a range if minimal changes are observed; data from toxicity-screening test should be reported if a bioaccumulation test was not conducted.

4.2.1.7 Test results

- for each treatment – mean ± SD for percent survival in a bioaccumulation test, unless not conducted in which case the percent survival, growth and observations from the toxicity-screening test are reported;
- citation of study and/or results of other toxicity tests if data used in lieu of a toxicity-screening test;
- for each reference and test treatment – mean ± SD for tissue concentration of the COPCs (dry weight, wet weight, or lipid basis);
- wet to dry weight conversion factors (or vice versa) if used;
- for each test treatment – mean ± SD for BSAFs of the COPCs (dry weight basis or normalized to lipid and TOC);
- coefficient of variation (CV) for mean percent survival, tissue concentration, and BSAF;
- results of any 96-h LC50 (including its 95% confidence limits) performed with the reference toxicant(s) using the same batch of organisms, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests using the procedures and conditions herein; and
- anything unusual about the test, any problems encountered, and remedial measures taken.

4.2.2 Additional reporting requirements

The following is a list of items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

4.2.2.1 Test substance or material

- identification of person(s) who collected and/or provided the sample;
- records of chain of continuity and long-entry sheets; and
- conditions (e.g., temperature, in darkness, in sealed container) of sample upon receipt and during storage.

4.2.2.2 Test organisms

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;
- history of brood stock;
- description of culture conditions and procedures (e.g., facilities and apparatus, lighting, water source and quality, water pretreatment, water exchange rate and method, water temperature, type and quality of substrate) for cultures;
- procedures used to count, handle, sort, transfer, and sieve animals, and those to determine their mortality, condition, appearance, and behavior; and
- source and composition of food, procedures used to prepare and store food, feeding method(s), feeding frequency and ration.

4.2.2.3 Test facilities and apparatus

- description of laboratory's previous experience with this biological test method for measuring bioaccumulation of contaminants from sediment into freshwater organisms;
- description of system for providing lighting and compressed air, and for regulating temperature within test facility;
- description of test chambers and cover used; and
- description of procedures used to clean or rinse test apparatus.

4.2.2.4 Negative control sediment and test water

- procedures for pretreatment of negative control sediment (e.g., sieving, settling of sieved fines, formulation and aging if formulated) and test water (e.g., filtration, sterilization, reconstitution and aging if reconstituted, temperature adjustment, aeration rate and duration);
- type and quantity of any chemical(s) added to test water;
- storage conditions and duration before use; and
- measured characteristics of water before and/or at the time of commencement of test (e.g., hardness, alkanity, pH, conductivity, metals, pesticides, nutrients, etc).

4.2.2.5 Test method

- procedures used for mixing or otherwise manipulating test sediment before use;
- time interval between preparation and testing;
- procedure used in preparing stock and/or test solution of chemicals;

- methods used (with citations) for chemical analyses of test material (sediment, tissue, overlying water) including details concerning aliquot sampling, preparation, and storage before analysis; and
- use and description of range-finding tests.

4.2.2.6 Test conditions and procedures

- measurement of light intensity adjacent to surface of overlying water in test chambers;
- statement concerning any aeration of overlying water in test chambers before and during the test;
- aeration rate and manner;
- records of any disruption of air flow to test chambers during the test, and of related DO measurements;
- appearance of each sample (or mixture thereof) and of the overlying water in test chambers; changes in appearance noted during test;
- physiochemical characteristics of negative control sediment(s) including sediment particle size, moisture content, density, TOC, and COPCs;
- for each sample – all measurements of sediment particle size, percent moisture content, and density;
- any other chemical measurements (e.g., contaminant concentration, acid volatile sulphides, biochemical oxygen demand, total inorganic carbon, cation exchange capacity, redox potential, pore water hydrogen sulphide, pore water pH and ammonia) made before and during the test (including negative control and reference sediment) and contents of test chambers, including analyses of whole sediment, pore water, and overlying water;
- any observations or analyses made on the test material (including samples of negative control and reference sediment), e.g., faunal tracks, qualitative and/or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses; and
- chemical analyses of concentrations of chemical in test solutions of reference toxicant.

4.2.2.7 Test results

- results from toxicity-screening tests or from standard toxicity tests if used in lieu of a toxicity-screening test for the same species;
- for each treatment – mean ± SD for percent growth (and change in lipid content where applicable) in a bioaccumulation test;
- CV for mean percent growth (and change in lipid content where applicable);
- tissue concentration of the COPCs for pre-exposure and negative control organisms (and lipid content where applicable);
- lipid content of organisms in reference and test treatments;
- warning chart showing the most recent and historic results for lipid content in pre-exposure organisms;
- results for any range-finding tests conducted;
- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);
- graphical presentation of data; and

- original bench sheets and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

Chapter 5: Additional Testing and Appendices

5.1 Reference Toxicant Tests

5.1.1 Use of tests

A reference toxicant is a chemical used in toxicity testing to make comparisons between test results for quality control purposes. The function of a reference toxicant test is to assess the relative sensitivity of the cultures (or batches) of test organisms and the precision of a test method. This incorporates all aspects of the method such as: test organism culturing and health; physical test conditions (lighting and temperature); gravimetric and volumetric measurements; as well as the technical proficiency of the analyst.

A static, 96-h, water-only test with a reference toxicant is presently recommended for organisms used in sediment toxicity methods (EC 1997 and 2013). Unlike methods which use organisms in the water column, reference toxicant tests with sediment organisms are not representative of the actual test method since they do not include sediment. Spiking negative control sediment with a reference toxicant is a possible method for conducting a reference toxicant test (EC 1995), but numerous problems have been encountered while trying to develop a standard method. Not only does this require more effort, but it can be costly, and does not provide consistent results. If different negative control sediments are spiked with a toxicant, it is unlikely that the variability associated with organism health, or technical proficiency could be separated from that due to different sediment characteristics (EC 1990). Formulated sediment could be used to standardize exposures, but it has been found that while test acceptability criteria for survival is usually met, growth is often significantly lower than in field-collected negative control sediment (Kemble et al. 1999). Since the recommended water only reference toxicant tests do not include sediment they are shorter in duration and an artificial substrate is often provided.

At present, a static, 96-h, water-only reference toxicant test is also recommended to assess the relative sensitivity and health of bioaccumulation test organisms. Selection of appropriate chemical(s) to use as a reference toxicant(s), general procedures, and interpretation of data are discussed in EC (1990). Reference toxicant tests should be run in conjunction with a bioaccumulation test or with each new batch of organisms, but may be performed on a monthly basis if organisms are cultured ‘in house’ and bioaccumulation tests are conducted routinely. The results of the most recent reference toxicant test should be used to assess and confirm the health of culture organisms before their use in a toxicity-screening or bioaccumulation test. Refer to Appendix D for an example of reference toxicant data and a control chart.

5.1.2 Test conditions and procedures

General test conditions in terms of temperature and lighting for reference toxicant tests are the same as for the toxicity-screening and bioaccumulation tests. Listed below are the specific procedures for conducting a static, 96-h, water-only reference toxicant test for the different test species. These test conditions and procedures are summarized in Table 6.

The recommended procedures for the invertebrate species are similar to the reference toxicant tests used for standard sediment toxicity test organisms (EC 1997a,b). The procedures for fathead minnows use larger test chambers and volumes of test solutions. Potassium chloride (KCl) is recommended as a reference toxicant, but other chemicals can be used.

There are 10 organisms per test chamber, with 5 test concentrations plus a negative control exposure. One or more replicates may be used. Recommended test chambers are glass jars or beakers with a capacity of > 300 ml for invertebrates and > 1500 ml for fathead minnows (~7 or 11 cm inner diameter, respectively). Reference test solutions are usually prepared by making up a solution of the highest test concentration (h.t.c.) and diluting by a factor of ≥ 0.5 to achieve the concentration series. The following h.t.c. and dilution factors are recommended for each test species;

Lumbriculus variegatus: 2 g/L KCl as the h.t.c., recommended 0.7 dilution factor

Hexagenia spp.: 10 g/L KCl as the h.t.c., recommended 0.5 dilution factor

Fathead minnow: 5 g/L KCl as the h.t.c., recommended 0.5 dilution factor

A stock concentration that also serves as the h.t.c. should be prepared using appropriate control/dilution water (section 2.2.4). It is recommended to save some of the stock (h.t.c.) for analytical confirmation if the results are outside the warning limits. The dilution series should be prepared dispensing solutions of each test concentration into test chambers (e.g., 200 ml for invertebrates, 1500 ml for fathead minnows). Water quality parameters (i.e., temperature, DO, pH, conductivity) should be measured for each test concentration (either on a subsample or in test chambers before the addition of organisms). No substrate is provided for *L. variegatus*[61] or fathead minnows, but tubes made of non-reactive material (e.g. silicone or polished glass; min 10, 5-10 cm long, ≥ 3.5 mm inner diameter) or clean nitex mesh should be added to test chambers for *Hexagenia* spp. *Hexagenia spp.* reference toxicant tests should be run in the dark or in opaque containers to avoid stress due to light exposure.

Test organisms used to begin the reference toxicant test should have an average wet weight of 5 ± 1 mg for *Hexagenia* spp., and 250-400 mg for fathead minnows. *L. variegatus* should be synchronized seven days[62] prior to testing to reduce variability in the test results (OECD 2007). Synchronization involves the dissection of large adult worms in the median body region with a scalpel. The posterior ends are retained in a vessel with culture substrate and water until exposure. Head regeneration in synchronized worms is indicated by burrowing into the substrate. For further details consult Annex 5 of OECD (2007).

Organisms should be separated from cultures and added to test chambers in a similar manner as with the toxicity-screening tests (section 3.2.3.2).

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[61] No apparent difference was observed in potassium chloride reference toxicant LC50 between tests run with quartz sand, coarse Nitex® mesh and without substrate (n=5). Oligochaetes weaved themselves throughout the Nitex® mesh making it difficult to remove organisms when they died.

[62] OECD (2007) recommends synchronization occurs 10-14 days prior to the start of exposure when reproduction is an endpoint of interest.

Organisms must not be fed during the test. Aeration of test solutions should be provided for tests with fathead minnows. On a daily basis, temperature should be measured. Mortalities[63] occurring during the test should be noted and dead organisms removed. If complete mortality occurs in a test chamber, water quality parameters should be measured on that day of the test.

The test should be terminated at 96 ± 2 h. The number of mortalities in each test chamber should be recorded and water quality measured. Surviving organisms should be euthanized and disposed of. Survival must be ≥ 90% in control exposures for the test to be valid.

The LC50 (estimated concentration killing 50% of the organisms) should be calculated following methods outlined in (EC 1990) and plotted against the mean and warning (2 SD) and control limits (3 SD) of data from the last 20 tests. If the LC50 from the test exceeds the warning limits it may be necessary to confirm the h.t.c. through chemical analysis. Analysis and interpretation of data from reference toxicant tests, including warning limits, confidence limits, and data trends are discussed in EC (1990).

[63] Mortality in invertebrates is indicated by a failure to move, despite gentle prodding or pushing water towards the organism. A colour change is also associated with death in *L. variegatus.* Oligochaetes tend to clump together making it difficult to assess partial mortality. While they should not be teased apart until test completion, gently pushing water through a pipette in the direction of the clump may loosen clumps of organisms.

Table 6. Summary of recommended condition and procedures for a reference toxicant test.

Parameter	Recommended Condition and Procedures
Test species	- *Lumbriculus variegatus*, *Hexagenia* spp., or fathead minnows
Test duration and type	- 96-h, static water-only test
Reference toxicant	- Potassium chloride (KCl), or other suitable chemical
Frequency of test	- once a month or in conjunction with sediment test or with each new batch of organisms
Test concentrations	- 5 plus a control; dilution factor should be ≥ 0.5
Control/dilution water	- uncontaminated surface water, groundwater, dechlorinated tap water, or reconstituted water; 90-100 % DO saturation
Organism test size (average wet weight)	- *L. variegatus* 6.5 ± 1 mg, *Hexagenia* spp. 5 ± 1 mg, fathead minnows 250-400 mg
No. organisms/chamber	- 10
Test chamber	- 400- or 1800[a]-ml glass jars (~7 or 11 cm diameter)
Volume of water	- 200 or 1500 [a] ml
Substrate	- none for *L. variegatus* or *P. promelas*; polished glass tubes (5-10 cm long, ≥ 3.5 mm inner diameter) for *Hexagenia* spp.
Replicates	- 1 or more
Temperature	- 23 ± 2°C daily average, ± 3°C instantaneous
Lighting	- 16 h light, 8 h dark, 500-1000 lux full spectrum fluorescent
Aeration	- only for fathead minnows
Feeding	- none
Observations/ measurements	- test initiation and termination: temperature, DO, pH, conductivity - daily: temperature, mortality
Endpoints	- survival, LC50 ± 95% confidence limits
Test performance-based acceptability criteria	- mean survival ≥ 90% in control exposures, LC50 ≤ 2 SD from the mean of the last 20 tests
[a] larger exposure chambers and volumes are for fathead minnows only	

5.2 Testing with spiked sediment

This protocol was developed for use with field-contaminated sediments, but may be adapted as necessary for use with spiked sediments. Guidance on procedures for spiking sediment and conducting toxicity tests is provided by EC (1995) and OECD (2007).

If less biomass is required due to the analytical procedures used, the size range of organisms (specifically *Hexagenia* spp.) can be narrowed and growth might be considered a sensitive endpoint. Increased replication and replication of control exposures might be necessary, and more easily achieved, for statistical comparisons depending on the objectives of the study.

Equilibration time is an important consideration in the assessment of bioaccumulation potential of a spiked-sediment and the maximum sediment storage time of eight weeks recommended by this method does not apply to testing of spiked-sediments (Kukkonen and Landrum 1998).

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Appendix A: Additional information regarding bioaccumulation tests and the selection of test species

Bioaccumulation should be considered on a case by case basis after initial toxicity-screening of the site (e.g., sediment sampling, modeling potential for exposure, fish collections, and caged mussel studies). In this way, laboratory bioaccumulation tests can focus on particular areas of contamination within the site or data gaps. While the choice of which species to use in a bioaccumulation test may depend on the scope, objectives, and budget of the project, some guidance or recommendations are offered below and should be discussed with the customer.

Situation/scenario	Comment
collect sufficient volume of sediment in field	- toxicity and bioaccumulation tests with multiple species; volume of sediment is not the limiting factor
collect young-of-the-year fish or benthic invertebrates with sediment	- validation of laboratory data with fish and invertebrate comparison
conduct toxicity tests before bioaccumulation tests	- longer holding time for bioaccumulation tests[64]; can focus on samples with low to moderate toxicity
no field-based bioaccumulation data	- use fish in laboratory tests due to reduced workload; focus on certain samples with invertebrates
sample is very coarse (> 80% sand), dense, or flocculent	- *Hexagenia* may not be able to construct or maintain burrows[65]
PAHs	- metabolized, not accumulated by *Hexagenia* spp. and fish, but can be accumulated by *L. variegatus*
PCDD/Fs	- partially metabolized, lower accumulation in fish than in *Hexagenia* spp. and *L. variegatus* (VanGeest *et al.* 2011c)
DDT and metabolites	- congener- and species-specific accumulation - DDT not accumulated by worms and *Hexagenia* spp. - DDD partially metabolized by *Hexagenia* spp.

[64] Refer to section 2.4.4.

[65] *Hexagenia spp.* exposed to a seven concentration dilution series of control sediments creating a range of particle size and TOC revealed nymphs appear to be tolerant of sediment up to 80% sand, if the remaining particles are silt and clay. Increased growth with decreasing sand and increasing TOC ($p \leq 0.05$) was observed. Survival was 100% and growth 98% in sediment with 78% sand and 0.4% TOC.

Appendix B: Use of Loss-on-Ignition (LOI) as a Surrogate Measure for Total Organic Carbon (TOC)

In the field of soil science, loss on ignition (LOI) is a commonly suggested alternative to dry combustion measurement of total organic carbon (TOC) due to its reduced cost and labour-requirements. Since LOI is a measurement of all organic matter, a relationship between LOI and TOC must be determined. The various approaches to predicting TOC based on LOI include the use of conversion factors and linear regression with and without intercepts, and have been reviewed by Sutherland (1998) and De Vos et al. (2005). Both authors caution against transferring generic forms of these models derived from one area to another environment. Therefore, historical sediment data from MOECC (2001-2008) were compiled to determine a relationship between TOC and LOI in typical sediment samples.

Only TOC and LOI measurements from unaltered samples were included in the data set. Samples that had been diluted with 'clean' negative control sediment were excluded as the TOC and LOI values are an artefact of two (or more) sediments. All data is held on file at the MOECC. The data was fit with a linear regression model in the form TOC = slope*LOI ± intercept. As discussed by Sutherland (1998) and De Vos et al. (2005), forcing the intercept through the origin (0,0) applies a bias that ignores experimental error, and is not recommended.

The resulting equation based on n = 198 sediment samples was *TOC (mg/g dw) = 0.4991 x LOI (mg/g dw) + 2.8487* with R^2 = 0.939 (Figure A). Some sediment samples with very high levels of organic contaminants tended to have measured TOC greater than that predicted by the equation. Approximately 96% of all sediment samples had TOC values ≤ 160 mg/g (Figure B) and the resulting model is likely most accurate for values within this range.

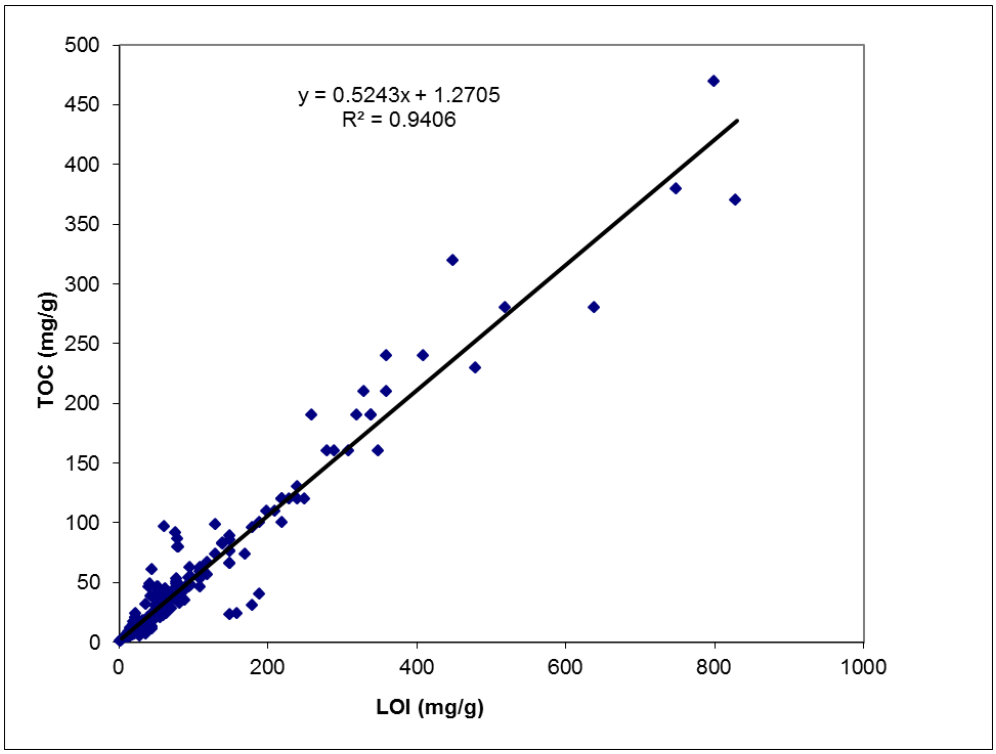

Figure A. Total organic carbon (TOC) versus loss on ignition (LOI) measured in historical MOECC sediment samples (2001-2015). Linear regression equation based on n = 230.

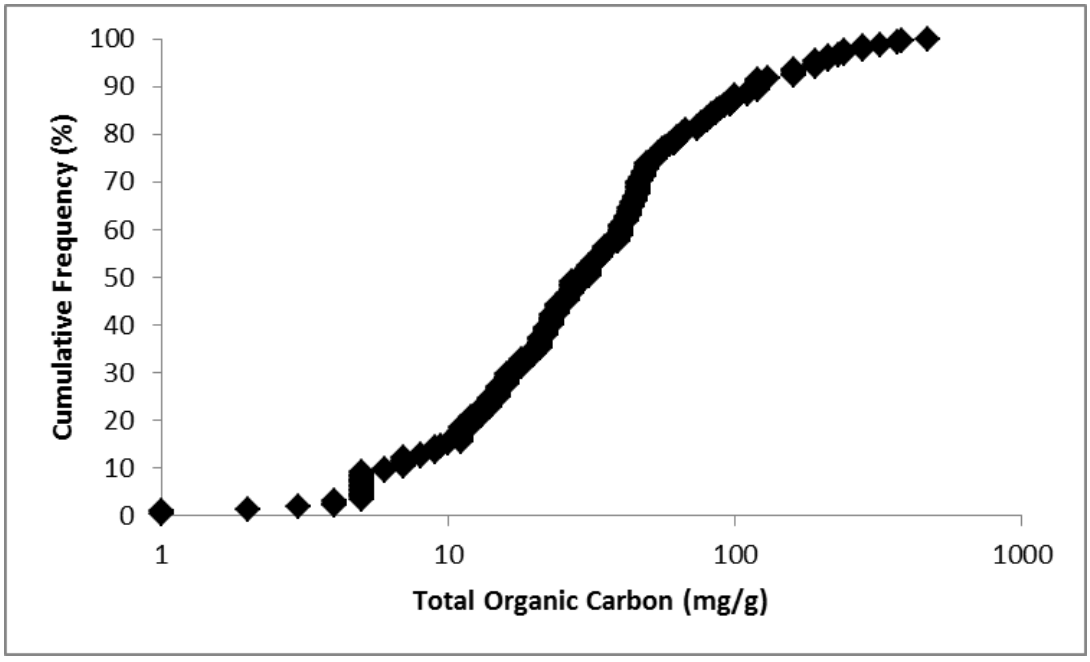

Figure B. Frequency distribution of total organic carbon (TOC) measured in historical MOECC sediment samples (2001-2015; n = 230).

Appendix C: Sources of uncertainty and estimated error (expressed as coefficient of variation – CV) in bioaccumulation testing during method development

Sources of uncertainty	% CV		
Sample handling and transport: Responsibility of sampler	NA		
Sediment homogenization:			
physical parameters: TOC and particle size	1-11		
chemical parameters (see method validation data)	13-24		
Measurements used to calculate sediment volume in test: (rounding volumes negates this error)			
Density: volumetric - 100 ml Teflon beaker	10		
Density: gravimetric - benchtop balance	0.01		
Density: based on dry weight	1-17		
Organic carbon: LOI measured as surrogate	0.5-3		
Organic carbon: TOC calculated from LOI (1-R^2 of linear regression)	6		
Organic carbon: TOC measured (OC = TC - CC)	3		
Dry/wet weight of culture organisms:	1.5 -10		
Test set up:			
Sediment distribution: volumetric - 100-250 ml Teflon beaker	10		
Water distibution: volumetric - 2000 ml graduate cylinder	5		
Organism distribution: gravimetric - microbalance and benchtop	0.01-10		
Organism distribution: variation in average wet weight	HX 34-83, FM 8-38		
Waterbath temperature: - thermometer calibration record	1°C		
Test termination:			
Organism biomass: gravimetric - benchtop balance	1		
Chemical analyses:			
Sediment:	varies per analyte, see analytical method uncertainty		
Biota:			
Lipid: (based on a reference value of 5% lipid)	17		
Biological variability:	LV	HX	FM
Lipid content of pre-exposure (d-0) organisms: (over time)	30	55	29
Lipid content of control (d-28) organisms: (over time)	47	30	26
Lipid content of test (d-28) organisms: (within test)	4-55	0-49	3-23
Concentration of contaminant(s) in biota[66]:			
DDD/DDE (ww)	9-35	0-17	3-51
PAHs (ww)	28-45	NA	NA
PCBs - total congeners (ww)	10-25	7-28	4-41

[66] Based on concentration/wet weight. Lipid normalization of contaminant concentrations reduced the CV in only 9%, 18 and 56% of cases (*L. variegatus* (n=11), *Hexagenia spp.* n=11) and *P. promelas* (n=16), respectively).

dl-PCB (ww)	9-37	5-20	8-12
PCDD/Fs (ww)	2-20	8-29	8-11
Hg (ww)	127	36	58
As (dw)	46	31	44

LV- *Lumbriculus variegatus*, HX - *Hexagenia* spp., PP - *Pimephales promelas*

Appendix D: Example of control chart of *Hexagenia spp.* method performance through observations of survival in negative control sediment over time.

Control charts, sometimes called Shewart charts or “process control charts” (ISO 1991), are a statistical tool used to measure how well you are meeting you performance goals. In toxicity testing these goals usually involve aspects of the test system which impact on the precision of your test results. Control charting water quality parameters (e.g. chlorine if the lab is using municipal water supply), test animal culture health parameters (e.g. lipid content or contaminant burden), control animal survival or the results from routine reference toxicant testing can identify ways to maintain consistency, and offer opportunities to improve method performance and data quality. For example, charting weekly chlorine measurements may indicate a trend of rising chlorine values in your culture dilution water, which allows you to perform maintenance on the water treatment system before the chlorine causes toxicity in your animal cultures or poor test performance. Charting control animal survival can demonstrate effectiveness of method performance and allow for increased sensitivity to discern toxicity in exposures (see Figures C and D).

Control charting reference toxicant test results is particularly effective in providing information on the combination of all aspects of the test methodology, including water quality, test system (e.g. lighting and temperature), organism health, volumetric and gravimetric measurements, technical training and performance, among others. This information, in graphical form, can indicate method uncertainty and detect trends that could impact data quality.

Control Survival Data Summary: *Hexagenia spp.*

raw | mean of last 20

ENTER NEW LC50 DATA HERE

year.proj # of Test	avrge survival %	test SD	avrg - 1SD	avrg+ 1SD	Mean	STD.DV.	- X-2SD LWL	+ 2SD UWL	- 3SD LCL	+ 3SD UCL	% CV	Control Chart Calculations Latest Mean	Latest LWL	Latest UWL	Latest LCL	Latest UCL	Latest CV
05.1	90	10	80.00	100.00	90.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!						
05.2	97	6	90.89	102.44	93.3	4.71	83.91	102.76	79.19	107.48	5.1						
05.3	87	23	63.57	109.76	91.1	5.09	80.93	101.29	75.84	106.39	5.6						
05.4	100	0	100.00	100.00	93.3	6.09	81.16	105.50	75.08	111.59	6.5						
05.5	96	7	89.07	102.93	93.9	5.40	83.06	104.67	77.66	110.08	5.8						
05.6	100	0	100.00	100.00	94.9	5.44	84.00	105.78	78.56	111.22	5.7						
06.1	93	6	87.56	99.11	94.7	5.00	84.66	104.67	79.66	109.68	5.3						
06.2	100	0	100.00	100.00	95.3	5.00	85.33	105.34	80.33	110.34	5.2						
07.1	100	0	100.00	100.00	95.9	4.93	85.99	105.71	81.06	110.64	5.1						
07.2	93	6	87.56	99.11	95.6	4.72	86.17	105.03	81.45	109.75	4.9						
07.3	100	0	100.00	100.00	96.0	4.67	86.67	105.33	82.00	110.00	4.9						
07.4	97	6	90.89	102.44	96.1	4.45	87.15	104.96	82.69	109.42	4.6						
08.1	100	0	100.00	100.00	96.4	4.40	87.55	105.16	83.15	109.57	4.6	98.2	92.5	103.8	89.7	106.6	2.9
08.2	98	4	93.53	102.47	96.5	4.25	87.97	104.98	83.72	109.23	4.4	98.2	92.5	103.8	89.7	106.6	2.9
09.1	100	0	100.00	100.00	96.7	4.20	88.32	105.11	84.12	109.30	4.3	98.2	92.5	103.8	89.7	106.6	2.9
09.2	97	6	90.89	102.44	96.7	4.05	88.60	104.82	84.54	108.87	4.2	98.2	92.5	103.8	89.7	106.6	2.9
09.3	100	0	100.00	100.00	96.9	4.01	88.89	104.92	84.88	108.92	4.1	98.2	92.5	103.8	89.7	106.6	2.9
09.4	100	0	100.00	100.00	97.1	3.95	89.16	104.98	85.21	108.94	4.1	98.2	92.5	103.8	89.7	106.6	2.9
09.5	100	0	100.00	100.00	97.2	3.90	89.42	105.03	85.52	108.93	4.0	98.2	92.5	103.8	89.7	106.6	2.9
09.6	100	0	100.00	100.00	97.4	3.85	89.67	105.06	85.82	108.91	4.0	98.2	92.5	103.8	89.7	106.6	2.9
09.7	100	0	100.00	100.00	97.5	3.79	89.90	105.08	86.11	108.87	3.9	98.2	92.5	103.8	89.7	106.6	2.9
09.8	100	0	100.00	100.00	98.0	3.42	91.14	104.80	87.72	108.21	3.5	98.2	92.5	103.8	89.7	106.6	2.9
10.1	96	5	90.52	101.48	97.9	3.43	91.07	104.80	87.64	108.23	3.5	98.2	92.5	103.8	89.7	106.6	2.9
10.2	98	4	93.53	102.47	98.5	2.26	93.95	103.00	91.69	105.26	2.3	98.2	92.5	103.8	89.7	106.6	2.9
11.1	96	5	90.52	101.48	98.3	2.30	93.69	102.88	91.40	105.17	2.3	98.2	92.5	103.8	89.7	106.6	2.9
13.1	98	4	94.00	102.00	98.4	2.24	93.91	102.85	91.67	105.09	2.3	98.2	92.5	103.8	89.7	106.6	2.9
13.2	100	0	100.00	100.00	98.4	2.24	93.91	102.85	91.67	105.09	2.3	98.2	92.5	103.8	89.7	106.6	2.9
13.3	96	9	87.00	105.00	98.5	2.00	94.51	102.51	92.51	104.50	2.0	98.2	92.5	103.8	89.7	106.6	2.9
14.1	100	0	100.00	100.00	98.5	2.00	94.51	102.51	92.51	104.50	2.0	98.2	92.5	103.8	89.7	106.6	2.9
14.2	100	0	100.00	100.00	98.5	2.00	94.51	102.51	92.51	104.50	2.0	98.2	92.5	103.8	89.7	106.6	2.9
15.1	98	4	94.00	102.00	98.7	1.62	95.49	101.97	93.88	103.58	1.6	98.2	92.5	103.8	89.7	106.6	2.9
15.2	88	8	80.00	96.00	98.2	2.82	92.52	103.80	89.70	106.62	2.9	98.2	92.5	103.8	89.7	106.6	2.9

Figure C: Example of data input into a control chart of *Hexagenia spp.* method performance through observations of survival in negative control sediment over time. The last 20 points are used to calculate latest mean, lower warning limit (LWL), upper warning limit (UWL), lower confidence limit (LCL), upper confidence limit (UCL) and coefficient of variation (CV). Warning limits are calculated as the mean±2*standard deviation (sd) and confidence limits are calculated as the mean±3*sd.

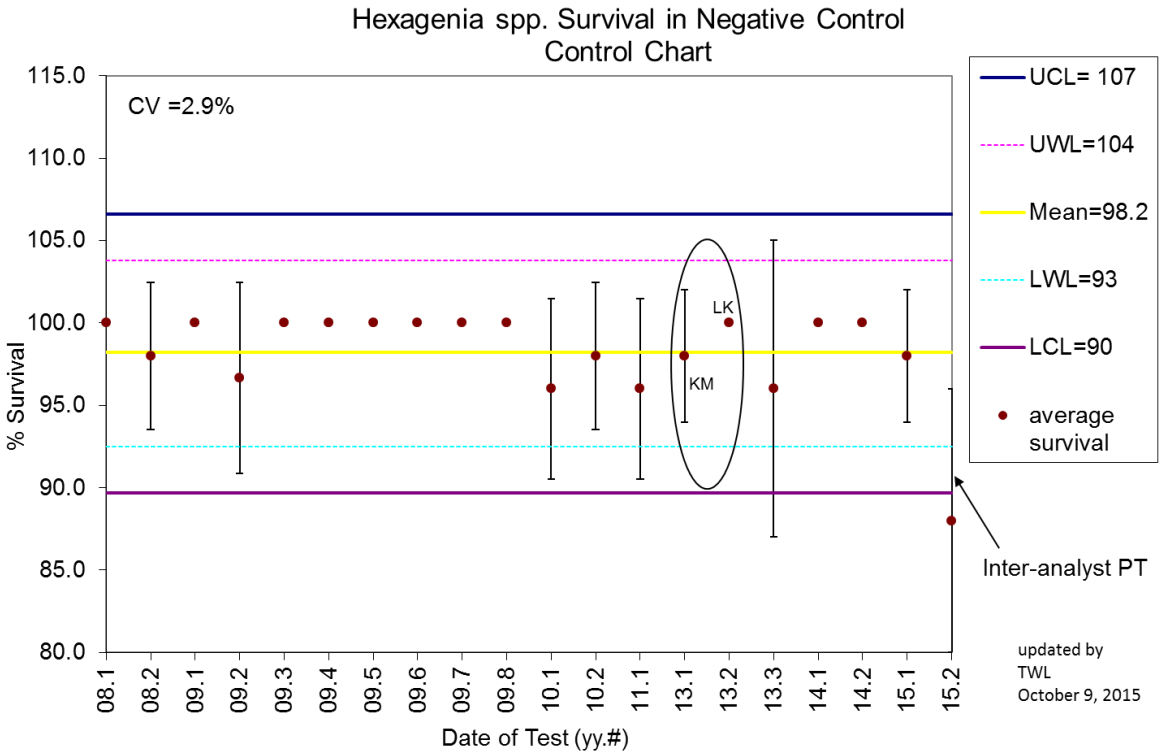

Figure D: Graphical example of a control chart of *Hexagenia spp.* method performance through observations of survival in negative control sediment over time.

PT = proficiency test