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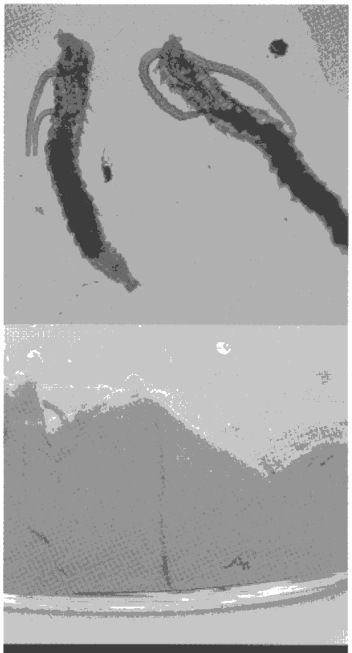

ENVIRONMENTAL
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Biological Test Method: Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)

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Review Notice

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Abstract

Procedures recommended by Environment Canada for performing sediment toxicity tests using spionid polychaete worms (Polydora cornuta) *are described in this biological test method. The species of worm selected for this test is a deposit-feeding, tube-dwelling, infaunal worm of the family Spionidae, which is found in estuarine or marine sediment within Canada's Atlantic coastal waters. The endpoints for the test are survival and weight of juvenile, laboratory-cultured worms. Test duration is 14 days.*

The test is conducted at 23 ± 1°C in 300-mL "high form" glass beakers containing a 50-mL (~2-cm) layer of sediment and 200 mL of overlying water. A minimum of five replicate test chambers, each containing five worms, are normally used for each treatment. The test may be run as a single-concentration assay (e.g., using undiluted samples of field-collected sediment), or as a multi-concentration assay (e.g., a spiked sediment test with chemical/sediment mixtures) to determine the threshold of effect. Juvenile worms of a similar age (i.e., 3-4 weeks' post-release) are used to start the test. Worms in each test chamber are fed three times per week, using a 1:1 mixture of ground tropical fishfood flakes (e.g., TetraMarin™) and ground estuarine green algae (Enteromorpha *sp*.) *and a per-feeding rate of 2.0 mg dry feed per worm. Approximately 80% of the overlying water is renewed on Day 7.*

General or universal conditions and procedures are outlined for test preparation and performance. Additional conditions and procedures are stipulated that are specific to the intended use of the test. This test is suitable for measuring and assessing the toxicity of samples of field-collected sediment, sludge, or similar particulate material; or of sediment spiked (mixed) in the laboratory with chemical(s) or chemical substance(s), contaminated sediment, or other particulate material. Instructions and requirements are included on test facilities, sample collection, handling and storing samples, culturing test organisms, preparing sediment or spiked-sediment mixtures and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of a reference toxicant.

Résumé

Le présent rapport décrit la méthode recommandée par Environnement Canada pour évaluer la toxicité de sédiments à l'aide de vers polychètes spionides (Polydora cornuta)*. Ce ver endofaunique, détritivore et tubicole appartient à la famille des spionides et vit dans les sédiments estuariens ou marins des eaux canadiennes de la côte Atlantique. Les paramètres de mesure sont la survie et le poids d'organismes juvéniles élevés* in vitro. *La période d'essai est de 14 jours.*

L'essai se déroule à 23 ± 1 °C dans des béchers en verre de 300 mL (forme haute) contenant une couche sédimentaire de 50 mL (~ 2 cm) et une colonne d'eau de 200 mL. Pour chaque variante de l'essai, on a normalement recours à cinq répétitions de l'enceinte expérimentale, chacune contenant cinq vers. On peut faire porter l'essai sur une seule concentration (p. ex., utilisation d'échantillons non dilués de sédiment prélevé sur le terrain) ou, pour déterminer le seuil à partir duquel se manifeste l'effet, sur plusieurs concentrations (p. ex., utilisation en parallèle d'un sédiment chimiquement enrichi ou d'un mélange de sédiments). On utilise des vers d'âge semblable (soit de 3 à 4 semaines post-éclosion) pour débuter l'essai. Ces vers sont nourris, dans chacune des enceintes trois fois par semaine, d'une mouture à parts égales de flocons de nourriture pour poissons tropicaux (p. ex., TetraMarin^{MC}) et d'algue verte estuarienne (*de l'espèce* Enteromorpha*) à raison de 2,0 mg de matière sèche par ver. Environ 80 % de la colonne d'eau est renouvelée le 7^{e} jour.*

Outre les conditions et modes opératoires universels (généraux) à adopter dans le cadre de la préparation et de la réalisation de tels essais, le présent rapport fait état de conditions et d'étapes additionnelles directement reliées à l'objectif de l'utilisateur. L'essai décrit dans le présent rapport peut être utilisé pour évaluer et mesurer la toxicité d'échantillons de sédiments, de boues ou d'autres matières particulaires prélevés tels quels sur le terrain ou enrichis (mélangés) en laboratoire par l'ajout de produits ou de substances chimiques, de sédiments contaminés ou d'autres matières particulaires. On trouve également dans le présent document des instructions et des exigences relatives aux installations d'essai, au prélèvement, à la manipulation et à l'entreposage d'échantillons, à l'élevage des organismes d'essai, à la préparation des sédiments purs ou enrichis, au démarrage des essais, à l'obtention des conditions requises, à la manière de procéder aux observations et aux mesures, aux paramètres de mesure, aux méthodes de calcul ainsi qu'à l'utilisation d'un toxique de référence.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of test materials or substances under controlled and defined laboratory conditions. Recommended methods are those that have been developed and published by Environment Canada (EC), and are favoured:*

- *for use in Environment Canada and provincial environmental toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory protocol or standard reference method.*

The different types of tests included in this series were selected because of their acceptability for the needs of programs for environmental protection and conservation in Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity to aquatic or terrestrial life of specific test substances or materials destined for or within the environment. Depending on the biological test method(s) chosen and the environmental compartment of concern, substances or materials to be tested for toxicity could include samples of sediment or similar particulate material, chemical or chemical product, or, where appropriate, effluent, elutriate, leachate, or receiving water. Appendix A should be consulted for a listing of the biological test methods and supporting guidance documents published to date by Environment Canada's Method Development and Applications Section in Ottawa, Ontario.

Words defined in the Terminology section of this document are italicized when first used in the body of the report according to the definition. Italics are also used to emphasize these and other words, throughout the report.

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List of Abbreviations

ANOVA	analysis of variance
cm	centimetre(s)
CV	coefficient of variation
DO	dissolved oxygen (concentration)
g	gram(s)
h	hour(s)
ICp	inhibiting concentration for a (specified) percent effect
kg	kilogram(s)
L	litre(s)
LC50	median lethal concentration
LOEC	lowest-observed-effect concentration
mg	milligram(s)
mL	millilitre(s)
MSD	minimum significant difference
mm	millimetre(s)
N	nitrogen
NOEC	no-observed-effect concentration
PCB	polychlorinated biphenyl
SD	standard deviation
TOEC	threshold-observed-effect concentration
TM (^{TM})	trade mark
v:v	volume-to-volume
°C	degree(s) Celsius
µg	microgram(s)
µm	micrometre(s)
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
±	plus or minus
%	percentage or percent
‰	parts per thousand (salinity)

Terminology

Words defined herein are italicized when first used in the body of the report according to the definition. Italics are also used to emphasize these and other words, throughout the report.

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

Grammatical Terms

Must is used to express 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 or more environmental factors such as temperature. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental regulations or requirements for issuing a permit.

Estuarine is from a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Growth is the increase in size or weight as the result of proliferation of new tissues. In this test, it refers to an increase in dry weight.

Marine is from or within the ocean, sea, or an inshore location where there is no appreciable dilution of water by natural fresh water derived from land drainage.

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 measurement of certain biological or water quality variables, or the collection and testing of samples of sediment for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. With respect to test substances or materials, ten percent (10%) represents ten units or parts of substance or material diluted with sediment or water to a total of 100 parts. Depending on the test substance, concentrations can be

prepared on a weight-to-weight, weight-to-volume, or volume-to-volume basis, and are expressed as the percentage of test substance in the final sediment mixture or solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or 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-h day.

Pretreatment means treatment of a sediment sample, or portion thereof, before exposure of the test organisms.

Reference method refers to a specific protocol for performing a toxicity test, i.e., a biological test method with an explicit set of test procedures and conditions, formally agreed upon by the parties involved and described precisely in a written document. Unlike the present document and other multi-purpose (generic) biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations.

Salinity is the total amount of solid substance, in grams, dissolved in 1 litre of (sea)water; and is traditionally expressed as parts per thousand (‰). It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1998).

Terms for Test Materials or Substances

Artificial sediment refers to a synthetic (formulated) sediment, prepared in the laboratory using a specific formulation intended to simulate a natural sediment.

Batch refers to the total amount of a particular *test sediment* or *test water* prepared for use in a sediment toxicity test. Following its preparation, a *batch* of test sediment may be subdivided into replicate test chambers; and a *batch* of test water may be subdivided and used as the overlying water introduced to each test chamber and/or as the control/dilution water used in a *water-only* reference toxicity test. The term *batch* might also be applied to a discrete culture or a discrete group of test organisms.

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 discernible distress to the test organisms or a reduction in their growth or survival 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 an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity 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 due to basic test conditions (e.g., temperature, health of test organisms, or effects due to their handling).

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

Control sediment – see *negative control sediment*.

Dechlorinated (municipal) 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 has been purified by passing it through resin columns or a reverse osmosis system, for the purpose of removing ions such as Ca^{++} and Mg^{++}.

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

Material is the *substance* or substances from which a thing is made. A *material* might have heterogeneous characteristics, even after mixing. Sediment, soil, or surface water are considered herein as materials. Usually, the *material* would contain several or many *substance*s.

Negative control sediment means uncontaminated (*clean*) sediment which does not contain concentrations of one or more contaminants that could affect the growth, survival, or behaviour of the test organisms. This sediment may be natural, field-collected sediment from an uncontaminated site, or *artificial sediment* formulated in the laboratory using an appropriate mixture of uncontaminated sand, silt, and/or clay. This sediment must contain no added test material or substance, and must enable an acceptable rate of survival and growth of *P. cornuta* according to the test conditions and procedures described herein. The use of *negative control sediment* provides a basis for interpreting data derived from toxicity tests using test sediment(s), and also provides a base sediment for spiking procedures.

Overlying water is water placed over sediment in a test chamber. See *control/dilution water* and *test water*.

Pore water (also called *interstitial water*) is the water occupying space between sediment particles.

Positive control sediment means sediment which is known to be contaminated with one or more toxic chemicals, and which causes a predictable toxic response with the test organisms according to the procedures and conditions of the biological test method described herein. This sediment might be one of the following: a *standard contaminated sediment*; *artificial sediment* or *reference sediment* that has been spiked experimentally with a toxic chemical; or a highly contaminated sample of field-

collected sediment, shown previously to be toxic to *P. cornuta* using this biological test method and for which its physicochemical characteristics are known. The use of *positive control sediment* assists in interpreting data derived from toxicity tests using *test sediment*.

Product is a commercial formulation of one or more chemicals. See also *chemical*.

Reconstituted water is high purity deionized or glass distilled water to which reagent grade chemicals have been added. The resultant synthetic water should be free of contaminants and have the desired pH, alkalinity, and hardness characteristics. Reconstituted water can also be water to which commercially available dry ocean salt or brine has been added, in a quantity that provides the seawater salinity (and pH) desired for culturing organisms and for testing purposes (e.g., for a test using estuarine sediment).

Reference sediment is a field-collected sample of presumably *clean* (uncontaminated) *sediment*, selected for properties (e.g., particle size, compactness, 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 that is uninfluenced or minimally influenced by the source(s) of anthropogenic contamination but within the general vicinity of the site(s) where samples of test sediment are collected. One or more samples of *reference sediment* should be included in each series of toxicity tests with *test sediment*(s). This sediment might or might not prove to be toxic due to the presence of naturally occurring chemicals such as hydrogen sulphide or ammonia, or the unanticipated presence of contaminants from human influence at harmful-effect concentrations. The use of such (toxic) sediment as *reference sediment* in future toxicity tests should be avoided, unless the experimental design is cognizant of this and the investigator(s) wish to compare test results for this material with those for one or more samples of test sediment.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms to establish confidence in the toxicity data obtained for 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 and 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 test, to appraise the sensitivity of the organisms and the precision and reliability of results obtained by the laboratory for that chemical at the time the test material or substance is evaluated. 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 with spionid polychaete worms 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.

Sampling station means a specific location, within a *site* or sampling unit (depending on the study design), where the sample(s) of field-collected sediment are obtained for toxicity tests and associated physicochemical analyses.

Sediment is natural particulate material, which has been transported and deposited in water and then deposited on the sea floor. The term can also describe a material that has been experimentally

prepared (formulated) using selected particulate material (e.g., sand of particular grain size, bentonite clay, etc.) and within which the test organisms can burrow.

Site means a delineated tract of sediment that is being used or considered as a study area, usually from the perspective of it being contaminated or potentially contaminated by xenobiotics.

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 a 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, drilling mud, contaminated dredge spoil, sludge, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the sediment.

Spiking refers to the addition of a known amount of chemical(s), chemical product(s), or other test substance(s) or material(s) (e.g., a sample of sludge or drilling mud) to a natural or artificial sediment. The substance(s) or material(s) is usually added to *negative control sediment, reference sediment*, or another *clean* sediment, but sometimes to a contaminated or potentially contaminated sediment. After the addition ("spiking"), the sediment is mixed thoroughly. If the added test material is a *site* sediment, Environment Canada documents typically do not call this spiking, but instead refer to the manipulation as "dilution" or simply "addition". See also *spiked sediment*.

Standard contaminated sediment is a field-collected sediment for which contaminant concentrations are known, documented, and available (e.g., from the National Research Council of Canada); and one which has proven to be toxic to *P. cornuta* using the biological test method described herein.

Stock solution means a concentrated solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water to prepare the required strengths 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 the toxicity test with spionid polychaete worms. In some instances, the term also applies to any solid-phase sample or mixture thereof (e.g., *negative control sediment*, *reference sediment*, dredged material, or *spiked 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 wet sieving of control sediment, for preparing formulated sediment or mixtures of spiked sediment, or for sieving the contents of each test chamber at the end of the test), and as control/dilution water for *water only* tests with a reference toxicant.

Water-only (toxicity) test refers to a (toxicity) test which does not include any sediment or other solid-phase material (e.g., a *water-only* test with aqueous solutions of a *reference toxicant*).

Statistical and Toxicological Terms

Acute means within a short period (seconds, minutes, hours, or a few days) in relation to the life span of the test organism.

Battery of toxicity tests is a combination of several toxicity tests, normally using different species of test organisms (e.g., a series of sediment toxicity tests using *P. cornuta*, one or more species of marine or estuarine amphipods, and *Vibrio fischeri*).

Chronic means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

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 SD ÷ mean.

Endpoint means the measurement(s) or value(s) that characterize the results of a test (e.g., LC50, ICp, NOEC, LOEC). This term might also mean the reaction of the test organisms to show the effect measured upon completion of the test (e.g., death or dry weight attained).

Geometric mean is the mean of repeated measurements, calculated logarithmically. It is advantageous in that extreme values do not influence the mean as is the case for an arithmetic mean. The *geometric mean* can be calculated as the n^{th} root of the product of the "n" values, and it can also be calculated as the antilogarithm of the mean of the logarithms of the "n" values.

ICp is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test substance or material that causes a designated percent inhibition (*p*) compared to the control, in a quantitative biological function such as growth. For example, an IC25 could be the concentration estimated to cause a 25% reduction in dry weight attained at the end of the test by the test organisms, relative to that in the control. This term should be used for any toxicological test which measures a continuously variable effect, such as dry weight at test end, reproduction, or respiration.

Intermittent renewal describes apparatus or tests in which solutions or overlying water in culture or test chambers are/is renewed periodically. Synonymous terms are *batch replacement, renewed static, renewal, static renewal, static replacement*, and *semistatic*.

LC50 is the median lethal concentration, i.e., the concentration of substance or material in sediment (e.g., mg/kg) or water (e.g., mg/L) that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of mortalities in five or more test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50 for a *water-only* reference toxicity test with *P. cornuta*, or 14-d LC50 for a *survival-and-growth* sediment toxicity test using *P. cornuta*).

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

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test substance or material for which a statistically significant effect on the test organisms was observed relative to the control. For example, the LOEC might be the lowest concentration at which the dry weight of exposed organisms at test end was significantly less than that in the control groups.

Minimum Significant Difference (*MSD*) means the difference between values for individual treatments (in this test with spionid polychaete worms, the difference in average weights or average mortality) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by certain statistical tests including *Williams' test*, a standard statistical procedure.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test substance or material at which no statistically significant effects on the test organisms were observed. For example, the NOEC might be the highest test concentration at which an observed variable such as dry weight at test end did not differ significantly from weight in the control groups.

Precision refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from replicate measurements differ. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp.

Quantal effects in a toxicity test are those in which each test organism responds or does not respond. For example, an animal might respond by dying in or avoiding a contaminated test soil. See also *quantitative*.

Quantitative effects in a toxicity test are those in which the measured effect is continuously variable on a numerical scale. Examples would be dry weight of *P. cornuta* at test end. See also *quantal*.

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). In a toxicity test comprising five replicate samples of undiluted field-collected sediment taken from each of four sites (including a reference site) plus replicate samples of control sediment, 25 test chambers would be used. For each *treatment* (i.e., for a particular sediment-collection site, or for a particular concentration in a test with contaminant-spiked sediment), there would normally be a minimum of five test chambers or replicates. A *replicate* must be an independent test unit; therefore, any transfer of organisms or test material from one test chamber (replicate) to another would invalidate a statistical analysis based on the replication.

Replicate samples are field-replicated samples of sediment collected from the same sampling station, to provide an estimate of the sampling error or to improve the precision of estimation. A single sediment sample from a sampling station is treated as one *replicate*. Additional samples are considered to be additional *replicate samples* when they are treated identically but stored in separate sample containers (i.e., not composited).

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

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 which directly causes death within the test period.

TOEC is the threshold-observed-effect concentration. It is calculated as the *geometric mean* of NOEC and LOEC. In some other countries, the concentration calculated in this way might be called the MATC (maximum acceptable toxicant concentration). *Chronic value* and *subchronic value* are alternative terms that have been used elsewhere and might be appropriate depending on the duration of the test.

Toxic means poisonous. A toxic chemical or material can cause adverse effects on living organisms, if present in sufficient amount at the right location. *Toxic* is an adjective or adverb, and should not be used as a noun; whereas *toxicant* is a legitimate noun.

Toxicant is a toxic substance or material.

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

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

Treatment refers to a specific *test sediment* (e.g., *site* sediment or *reference sediment* from a particular sampling station and depth), or a concentration thereof. Samples or subsamples of test sediment representing a particular *treatment* are typically replicated in a toxicity test. See also *replicate.*

Warning chart is a graph used to follow changes over time, in the endpoints for a *reference toxicant*. Date of the test is on the horizontal axis and the effect-concentration is plotted on the vertical logarithmic scale.

Warning limit is plus or minus two standard deviations, calculated logarithmically, from a historic geometric mean of the endpoints from tests with a *reference toxicant*.

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This biological test method was co-authored by D. McLeay (McLeay Environmental Ltd., Victoria, BC) and P. Pocklington (Arenicola Marine, Dartmouth, NS). Statistical guidance herein was written or reviewed and improved by J.B. Sprague (Sprague Associates Ltd., Salt Spring Island, BC).

The document is based on existing technical reports that describe procedures and conditions used by Environment Canada's laboratory personnel for culturing spionid polychaete worms and for conducting sediment toxicity tests using these test organisms (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000; Fennell and Bruno, 1997, 1998). Other *survival-and-growth* tests for sediment toxicity published by Environment Canada (EC, 1997a, 1997b) have guided the development of this biological test method. Standard procedures and conditions for sediment toxicity tests using *Neanthes* sp., as developed for and recommended by the United States Environmental Protection Agency (USEPA, 1990a, b), the American Society for Testing and Materials (ASTM, 1994), and the United States Army Corps of Engineers (USACE, 1995) have also helped guide the preparation of this test method, as have the published United States procedures (USEPA, 1994a; ASTM, 1995b) for undertaking growth and/or survival tests for sediment toxicity using cultured species of freshwater invertebrates.

R.P. Scroggins (Method Development & Applications Section, Environmental Technology Centre, EC, Ottawa, ON) acted as Scientific Authority and provided technical input and direction throughout the work. A. Chevrier and L. Porebski (Marine Environment Branch, EC, Hull, PQ) served as senior technical advisors during the procedural and validation studies associated with developing this biological test method. The developmental studies resulting in this biological test method were undertaken at Environment Canada's Atlantic regional laboratory (Dartmouth, NS and, since March 1997, Moncton, NB) by T. Corbin, K. Doe, A. Huybers, P. Jackman, M. Pocklington, S. Wade, and G. Wohlgeschaffen; and at the Pacific & Yukon regional laboratory (North Vancouver, BC) by J. Bruno, M. Fennell, D. Lee, G. van Aggelen, and S. Yee. The interlaboratory studies undertaken to validate the biological test method (McPherson *et al.*, 1998) were coordinated by EVS Environment Consultants (C. McPherson) and performed by Environment Canada's Atlantic and Pacific & Yukon regional laboratories together with those of EVS Environment Consultants (North Vancouver, BC) and the USACE (Vicksburg, MS).

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Cover photos provided by P. Pocklington, K. Doe, and K. Bentham.

Introduction

1.1 Background

In the aquatic environment, *sediment* provides habitat for many organisms, while being a major repository for many of the more persistent *chemical*s that are introduced into surface waters. Research has shown growing evidence of environmental deterioration, however, including effects on aquatic organisms in or near sediment, even when federal or provincial water quality criteria are not exceeded. Other evidence shows that sediment can be contaminated with high concentrations of chemicals of *toxic* concern, yet a diverse community of benthic or epibenthic organisms is present and no harm to exposed aquatic life can be demonstrated.

Single-species *sediment toxicity test*s are cost-effective tools for determining whether contaminants in sediment are harmful to benthic or epibenthic organisms as well as those frequenting the overlying water column. They can be used for various purposes, including:

- determining the spatial and temporal distribution of contaminants in sediment that are toxic to benthic or epibenthic organisms;
- measuring the toxicity of dredged or other *material* being considered for disposal to surface waters;
- ranking aquatic environs adjacent to industrial sites for quality and/or cleanup requirements;
- appraising the environmental integrity of approved disposal sites;
- estimating the effectiveness of management or remediation practices;
- investigating interactions of environmental relevance between contaminants in sediment and *overlying water*;
- comparing the relative sensitivities of various benthic and epibenthic organisms;
- determining the relationship between toxic effects and bioavailability for contaminants in sediment; and
- establishing and applying sediment quality guidelines and objectives.

In the laboratory, results of toxicity tests with samples of *clean* (uncontaminated) sediment spiked with different concentrations of contaminants can be used to assess "cause-and-effect" relationships between chemicals and biological responses. Environmental appraisal of spatial (horizontal and/or vertical) and temporal sediment quality is most effective if a battery of appropriate single-species sediment toxicity tests is integrated with sediment chemistry (including bulk sediment and porewater analyses) and biological surveys.

Toxicity tests are used routinely within Canada and elsewhere to determine and monitor the toxic effects of discrete *substance*s or complex mixtures that might be harmful to indigenous aquatic life in the environment (water and sediment). The results of toxicity tests can be used to determine the need for control of discharges, to set environmental standards, and for research and other purposes. Recognizing that no single biological test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, Canada's Inter-Governmental Aquatic Toxicity Group (IGATG; now referred to as IGETG, see Appendix B) proposed a set of single-species

toxicity tests using selected marine, estuarine, or freshwater organisms which would be broadly acceptable for use in Canada. Tests chosen for development and standardization by Environment Canada included those which would measure different toxic effects using different test *substance*s or *materials* (e.g., samples of *chemical* or chemical product, effluent, receiving water, or sediment), different biological *endpoint*s (*lethal* or *sublethal*, *acute* or *chronic*), and organisms representing different trophic levels and taxonomic groups.

In 1987, Environment Canada and IGATG recommended that a consistent set of sediment testing methods be developed for routine use in preventing, appraising, remediating, and managing *contaminated sediment* (Sergy, 1987). At that time, Environment Canada's regional laboratories (see Appendix C for contacts) commenced a series of studies (McLeay *et al.*, 1989, 1991, 1992, 1993; Paine and McPherson, 1991a, b; Doe and Wade, 1992; Yee *et al.*, 1992) to develop and evaluate a standardized biological test method for measuring the toxicity of samples of contaminated sediment using one or more of six species of marine or estuarine amphipods common to Canadian Pacific or Atlantic coastal waters (EC, 1992a). New *reference methods* (i.e., standard protocols) for measuring sediment toxicity have been published (EC, 1998) (see Appendix A) or will be in the near future (EC, 2001a). Other biological test methods have also been standardized by Environment Canada for various applications including the measurement and appraisal of the toxicity of samples of whole sediment, *pore water*, or elutriate; and are available for widespread use (e.g., EC, 1992b, c). Guidance documents have been published describing suitable procedures for collecting and manipulating sediment samples, and for *spiking clean sediment* with chemicals in preparation for whole-sediment *reference toxicity test*s (EC, 1994, 1995). Toxicity tests which measure survival and growth (mean dry weight at test end) in sediment using either the freshwater amphipod *Hyalella azteca* (EC, 1997a) or a species of midge larvae (*Chironomus tentans* or *C. riparius*; EC, 1997b) have been published by Environment Canada as part of a series of biological test methods prepared to help meet Canadian requirements related to environmental appraisal and protection.

This generic (multi-purpose) biological test method provides guidance and specific procedures for conducting a test for sediment toxicity using a selected species of spionid polychaete worm (i.e., *Polydora cornuta*) common to Canada's Atlantic coastal waters. It can be applied to one or more samples of field-collected test sediment (see Section 5), or to *clean* sediment which has been spiked in the laboratory with one or more concentrations of a test *chemical* or chemical *product* (see Section 6). The test method can be performed as either a single-concentration test (e.g., using samples of field-collected sediment at 100% concentration only) or a multi-concentration test (e.g., *clean* sediment spiked with one or more chemicals or chemical products; or a range of concentrations of field-collected *test sediment* in *clean* sediment). The test uses laboratory-cultured organisms, and measures both survival and dry weight attained at test end as the biological responses. It is based largely on similar *survival-and-growth* tests for sediment toxicity to juvenile polychaetes (*Neanthes* sp.) that have been developed for use by the United States Environmental Protection Agency (USEPA, 1990a, b), the American Society for Testing and Materials (ASTM, 1994), and the United States Army Corps of Engineers (USACE, 1995) (see Section 1.2). A partial summary of the procedural specifics put forward by these agencies for culturing polychaete worms, and for performing sediment toxicity tests and related reference toxicity tests, is provided in Appendices D, E, and F. Other significant United States reports which have helped guide the development of this document include the biological test methods for measuring the toxicity of sediment-associated contaminants published in 1994 by the USEPA (1994a, b).

Universal procedures for preparing and conducting sediment toxicity tests using a selected species of spionid polychaete worm (i.e., *Polydora cornuta*) are described in this report. Also presented are specific conditions and procedures which are required or recommended when using the test for evaluating different types of *substances* or *materials* (e.g., samples of field-collected sediment or particulate waste, or samples of one or more chemicals or chemical products experimentally mixed into or placed in contact with natural or artificial sediment).

The flowchart in Figure 1 gives a general picture of the universal topics covered herein, and lists topics specific to testing samples of field-collected sediment, similar particulate waste (e.g., sludge, drilling mud, or dredged material), or sediment spiked experimentally with chemical(s) or chemical product(s), contaminated sediment, or particulate waste. This biological test method is intended for use in evaluating the toxicity of samples of the following:

(1) field-collected sediment from marine or estuarine waters;

(2) dredged spoils under consideration for ocean disposal;

(3) industrial or municipal sludge and similar particulate wastes that might affect the marine or estuarine environment; and

(4) laboratory-prepared mixtures of clean and contaminated sediments, or of one or more chemicals or chemical products within or overlying marine or estuarine sediment.

In formulating this generic (multi-purpose) biological test method, an attempt has been made to balance scientific, practical, and cost considerations, and to ensure that the results will be sufficiently precise for most situations in which they will be applied. It is assumed that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions that might be required in a *reference method* (i.e., a regulatory protocol) are not provided in this report, although it is intended as a guidance document useful for that and other applications.

For guidance on the implementation of this and other biological test methods, and on the interpretation and application of the endpoint data, the reader *should* consult Environment Canada (1999).

1.2 Historical Use of Polychaete Worms in Toxicity Tests

The use of polychaete worms for laboratory toxicity tests and as key organisms for marine environmental quality monitoring has gained in popularity during the past two decades (Reish, 1980a, b, 1985; Pocklington and Wells, 1992). This is deserving in many respects, and reflects:

- the world-wide distribution of large numbers and species of polychaetes in intertidal and subtidal sediment[1] (Berkeley, 1927; Farke and Berghuis, 1979; Bailey-Brock, 1984; Maurer and Reish, 1984; Brinkhurst, 1987; Pocklington, 1989);

- their use as "indicator" species in coastal surveys of environmental integrity and deterioration (Carr and Neff, 1984; Grassle and Grassle, 1976, 1984; Long and Chapman, 1985; Waldichuk, 1988; Zajac and Whitlach, 1988; Parrish *et al.*, 1989; Becker *et al.*, 1990; Power and Chapman, 1991; Pocklington and Wells, 1992);

[1] In general, polychaetes constitute over 40% of both the number of species and the specimens in subtidal, soft-bottom benthos, regardless of depth or latitude (Reish, 1980a).

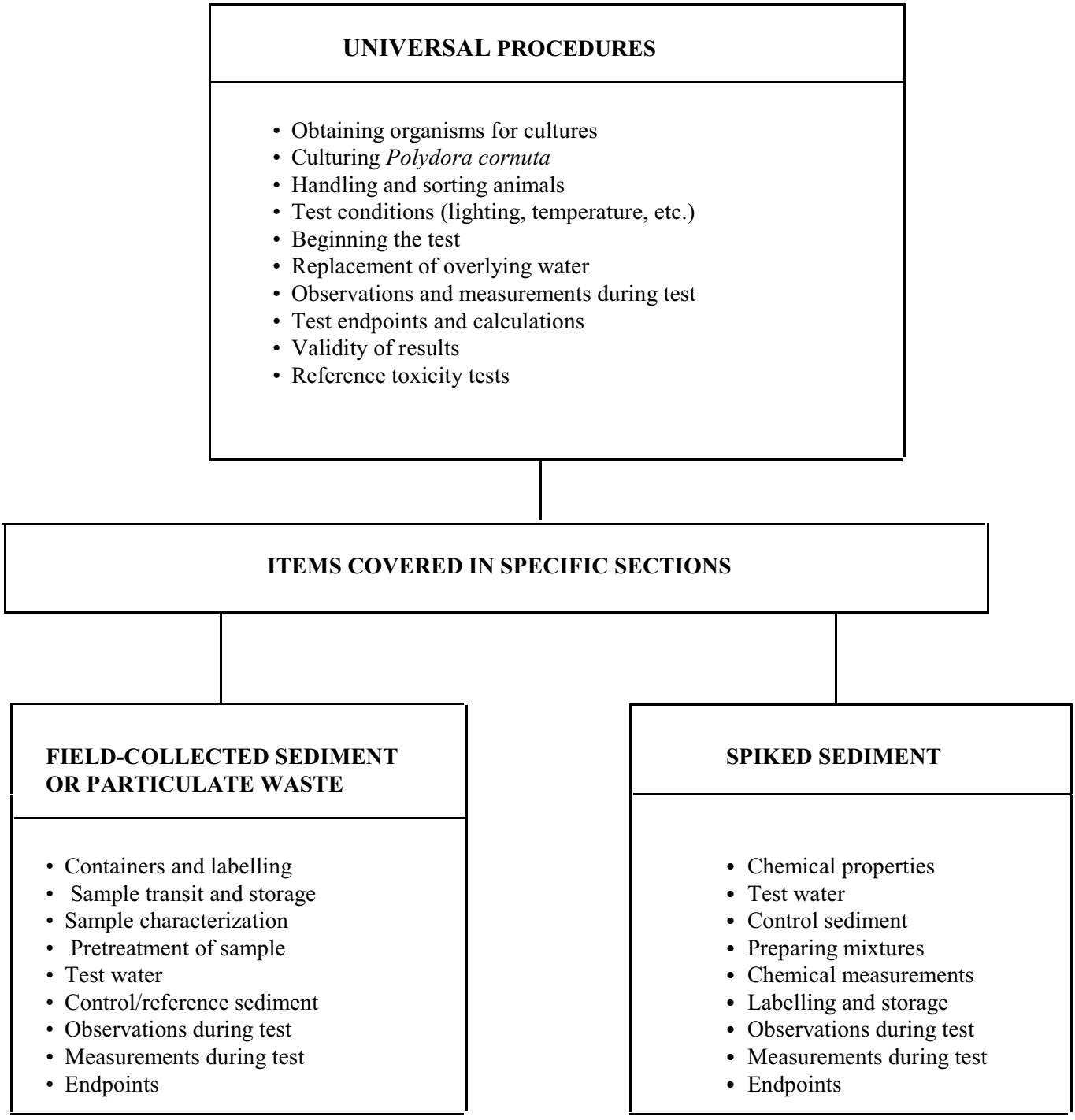

Figure 1 Considerations for Preparing and Performing Toxicity Tests Using Spionid Polychaete Worms (*P. cornuta*) and Various Types of Test Materials or Substances

- their ecological importance as prey and predators in the marine food web (Pocklington and Wells, 1992; ASTM, 1994);

- the growing body of historical information regarding their taxonomic features and biology (e.g., Herpin, 1926; Klawe and Dickie, 1957; Reish, 1957, 1970, 1977, 1979; Akesson, 1967; Reish and Alosi, 1968; Abati and Reish, 1972; Grassle and Grassle, 1974; Davis and Reish, 1975; Schroeder and Hermans, 1975; Rice and Reish, 1976; Fauchald and Jumars, 1979; Bailey-Brock, 1984; Pesch *et al.*, 1987, 1988; Gremare *et al.*, 1989a, b); and

- the sensitivity of certain species, life stages, and life processes of polychaete worms to aquatic and sediment-associated contaminants.

The demonstrated ability to culture various species of polychaetes in the laboratory (Reish, 1976, 1980a, b, 1981; Carr and Curran, 1986; Pesch and Schauer, 1988; Pocklington *et al.*, 1995); Corbin *et al.*, 2000) strengthens the worth of these species for year-round toxicity tests with samples of field-collected sediment or similar particulate material (Section 5), spiked-sediment mixtures (Section 6), sediment pore water, or aqueous contaminants in association with sediment.

Polychaete worms have been used extensively for measuring the toxicity of aqueous or solid-phase materials associated with marine or estuarine environments. Toxicity tests with these organisms have been frequently restricted to tests which measured the acute lethality of metals and/or organic contaminants in water or sediment (Carr and Reish, 1977; Pesch and Morgan, 1978; McLeese *et al.*, 1982; Reish and LeMay, 1991; Schiff *et al.*, 1992; Casas and Crecelius, 1994; Reish and Gerlinger, 1997). Such tests continue to serve a purpose, although in many instances the acute lethal tolerance of juvenile or adult life stages of polychaetes is high relative to that for other more sensitive marine or estuarine invertebrates such as amphipods. A variety of acute or chronic sublethal toxicity tests with polychaete worms have measured or examined the following:

- various adverse effects of aquatic contaminants in water or sediment, including biochemical or physiological changes (Abati and Reish, 1972; Cripps and Reish, 1973; Carr and Neff, 1982, 1984; Neuhoff, 1983; Johns *et al.*, 1985);

- histopathologies (Gentile *et al.*, 1990); avoidance or other behavioural effects (Rubinstein, 1979; Akesson and Ehrenstrom, 1984; Olla *et al.*, 1988; Rice *et al.*, 1994);

- genotoxic effects (Pesch *et al.*, 1981; Harrison *et al.*, 1984, 1987; Anderson and Harrison, 1986; Anderson *et al.*, 1987, 1990; Pesch, 1990);

- developmental anomalies (Foret, 1974; Reish *et al.*, 1974; Chapman *et al.*, 1985; Walsh *et al.*, 1986); and

- reproductive effects (Reish and Carr, 1978; Oshida *et al.*, 1981; Chapman and Fink, 1984; McCoppin-Frohoff, 1983; Reish and Gerlinger, 1984; Anderson *et al.*, 1987; Jenkins and Mason, 1988; Carr *et al.*, 1989; Johns and Ginn, 1990; Long *et al.*, 1990; Johns *et al.*, 1991a; Moore *et al.*, 1991; Pesch *et al.*, 1991; Carr and Chapman, 1992; Gerlinger *et al.*, 1993a; Moore and Dillon, 1993).

Numerous studies have also used polychaete worms to appraise the bioaccumulation of inorganic or organic contaminants from water or sediment, or the biomagnification of contaminants through the food chain (Neff *et al.*, 1978; Jenkins and Sanders, 1986; LeMay and Reish, 1988; Olla *et al.*, 1988; Mason *et al.*, 1988; Weston, 1990; Moore *et al.*, 1991; Reish and LeMay, 1991; USACE, 1993a). Chronic growth and/or survival tests for aqueous or sediment toxicity, which use deposit-feeding polychaetes as test organisms,

have been ongoing with increasing frequency for two decades or so (Anderson, 1977; Neuhoff, 1983; Pesch and Hoffman, 1983; Chapman *et al.*, 1985; Jenkins and Sanders, 1986; Johns and Ginn, 1989, 1990; Pastorok and Becker, 1990; Johns *et al.*, 1991b; Moore *et al.*, 1991; Pesch *et al.*, 1991; Tay *et al.*, 1992; Dillon *et al.*, 1993a, b; Moore and Dillon, 1993; Gerlinger *et al.*, 1993b, 1995; Rice *et al.*, 1994, 1995; Bridges and Farrar, 1997; Bridges *et al.*, 1997; Anderson *et al.*, 1998; Farrar *et al.*, 1998). These tests have been shown to be appreciably more sensitive to environmental contaminants than acute lethality tests with the same species. Comparative studies of growth and reproduction using *Neanthes arenaceodentata* have demonstrated that, for this species at least, there is a relationship between reduced growth and impaired reproductive success due to contaminated sediment (Johns *et al.*, 1991a) or reduced food rations (Moore and Dillon, 1993).

Considerable research efforts have been spent by United States researchers to develop and standardize a *survival-and-growth* test for sediment toxicity using the deposit-feeding, tube-dwelling polychaete *Neanthes arenaceodentata*. This work (e.g., Johns and Ginn, 1989, 1990; USEPA, 1990a) resulted in the publication of a standard protocol for conducting a 20-day *survival-and-growth* test for sediment toxicity using this species of polychaete (USEPA,1990b). The ASTM (1994) subsequently published a standard guide for conducting a 20- to 28-day test for survival and growth of *N. arenaceodentata* in *test sediment*s. Research by scientists at the USACE Waterways Experiment Station in Vicksburg, MS, as reported in an excellent series of technical reports (e.g., USACE, 1990, 1992, 1993a, b, c, d, e, 1994a, b) as well as in the primary literature (e.g., Dillon *et al.*, 1993a, b; Moore and Dillon, 1993), has resulted in the development by the USACE of a standardized, 28-day *survival-and-growth* test for the regulatory evaluation of dredged material, which uses *N. arenaceodentata* as test organisms (USACE, 1995).

In 1992, Environment Canada commenced a program of studies intended to develop and standardize a *survival-and-growth* test for sediment toxicity using one or more species of polychaete worms. Initial efforts focused on an identification of candidate test species common to Canada's coastal (Atlantic, Pacific, Arctic) waters[2], together with their rating as to potential suitability for this test.[3] Based on this survey, four Atlantic species (*Polydora cornuta, Clymenella torquata, Nephtys neotena, Nereis (Neanthes) diversicolor*), four Pacific species (*Boccardia proboscidea, Axiothella rubrocincta, Nephtys caecoides, Hediste limnicola*) and two Arctic species (*Polydora quadrilobata, Euchone papillosa*) were chosen for laboratory investigation (Arenicola Marine, 1992). A series of studies with field-collected specimens representing each of these 10 species was undertaken, including tests for survival and growth in *control* and contaminated sediments and in *water-only* reference toxicity tests with cadmium (Arenicola Marine, 1993, 1994). Based on the findings of these studies, culturing trials and related non-contaminant-effect and contaminant-effect tests were conducted using three polychaete species of the family Spionidae (i.e., *P. cornuta, B. proboscidea, P. quadrilobata*), and two species of the family Maldanidae (i.e., *C. torquata, A. rubrocincta*). These culturing trials and tests resulted in the selection of two spionid species (i.e., *P. cornuta* and *B. proboscidea*) as candidate test organisms, and the rejection of the third spionid species (*P. quadrilobata*) and the two maldanid species from further consideration (Pocklington *et al.*, 1995). The results of

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[2] *Neanthes arenaceodentata* is not found in Canadian waters.

[3] Considerations included available information regarding each species in the published literature, such as their known distribution, biology, ecological requirements, life history, rapid life cycle, adaptability to laboratory conditions, ability to culture these or similar species, sensitivity to contaminants, and tolerance to non-contaminant confounding factors such as sediment grain size and organic content.

subsequent culturing trials and tests (Corbin *et al.*, 1998, 2000; Fennell and Bruno, 1997, 1998; McPherson *et al.*, 1998) led to a decision by Environment Canada to reject *B. proboscidea* from further testing, and to restrict the 14-day *survival-and-growth* test for sediment toxicity described herein to a single species of spionid polychaete worm, i.e., *P. cornuta*.

1.3 Identification, Distribution, and Life History of P. cornuta

Polydora cornuta (Polychaeta: Spionidae) is representative of a widely distributed family of polychaetes — the Spionidae. This species belongs to the polydorid group of spionids, members of which are recognized by a structurally modified fourth or fifth setiger bearing at least one set of specialized setae. Taxonomic descriptions and illustrations of the distinguishing features of *P. cornuta* are given in Appendix G, together with information regarding its distribution and life history (i.e., ecology, reproduction, and development).

P. cornuta is found naturally in Canadian (Atlantic) coastal waters, and has been introduced to the Pacific coast of North America (Rice, 1975; Blake and Maciolek, 1987; Pocklington, 1989). Spionids in general have been reported to reproduce rapidly (Schroeder and Hermans, 1975), and are easily collected intertidally (e.g., to obtain organisms to start cultures). *P. cornuta* is found in fine, silty sand, where it builds a tube which extends about 0.5 cm above the sediment surface and about 2 cm below the surface. Using its palps, *P. cornuta* collects particles from the seabed and uses these particles either as food or as tube-building material. This species tolerates some degree of anoxic conditions in the sediment, probably because it can access overlying oxygenated water via its tube. Like other species of polychaetes, the spionids are an abundant and often dominant component of benthic communities found in sediments within marine or estuarine environments. They are an important source of food for larger invertebrates, as well as migrant or resident fish and shore birds. Spionids and other polychaete worms contribute significantly to biogeochemical processes in sediment through its bioturbation, reworking, and aeration. The rapid life cycle of *P. cornuta* (i.e., ~28 days, under laboratory conditions), small size, euryhalinity, ease of culturing, and rapid growth of juveniles under laboratory conditions (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000; Fennell, 1998; Fennell and Bruno, 1997, 1998; Jackman *et al.*, 1999) distinguish this species of spionid polychaete worm as one with characteristics sought when selecting a laboratory test organism suitable for routine use in *survival-and-growth* tests for sediment toxicity.

1.4 Laboratory Performance and Relative Sensitivity of P. cornuta

Spionid polychaetes are robust and can withstand a wide range of laboratory conditions. Results for non-contaminant-effect tests and contaminant-effect tests using field-collected *P. cornuta* are given in Arenicola Marine (1994); whereas those for similar tests with laboratory-cultured organisms of this species as well as the spionid polychaete *Boccardia proboscidea* have been summarized by McLeay *et al.* (1997), Corbin *et al.* (1998), and Jackman *et al.* (1999) and are presented in detail in Pocklington *et al.* (1995), Fennell (1998), McPherson *et al.* (1998), and Corbin *et al.* (2000).

P. cornuta can survive well in the laboratory. The survival rate was 80 % when field-collected specimens were held for 30 days in aerated seawater (28‰) in the absence of food and sediment; groups held for this period in negative control sediment and with feeding showed survival rates ranging from 92 to 100% (Arenicola Marine, 1994). Similarly, survival rates for groups of cultured juveniles fed in negative control sediment for periods of 14 to 20 days ranged from 92 to 100% when the overlying water was renewed weekly (Pocklington *et al.*, 1995).

One control experiment showed a lower survival rate (mean = 84%) when overlying water was not renewed.

Non-contaminant-effect tests with *replicate* groups of cultured worms held in negative control sediment for 20 days at each of three salinities (28, 15, and 5‰) showed mean survival rates of 100%, 95%, and 90%, respectively. Growth was unaffected by a *salinity* of 15‰, although it was decreased by approximately 50% for the replicate groups held in overlying water with a salinity of only 5‰ (Pocklington *et al.*, 1995). These results indicate that *P. cornuta* can be used in *survival-and-growth* tests with sediment whose porewater salinity is ≥15‰. Additional experiments by Corbin *et al.* (1998, 2000) with salinity-acclimated cultures have shown that *P. cornuta* can survive and reproduce at salinities ≥10‰; and that worms of this species survive and grow well when acclimated to 30‰ salinity and then held for 14 days in negative control sediment with porewater salinity ≥10‰. Animals cultured at 20°C and then tested for survival and growth in negative control sediment at either 20°C or 10°C showed a 50% decrease in mean dry weight of worms at test end for the lower test temperature, as well as a reduced 20-day mean survival rate at the lower test temperature (i.e., 100% at 20°C and 72% at 10°C) (Pocklington *et al.*, 1995). These results supported conducting sediment toxicity tests with this species at the higher temperature (20°C). Subsequent side-by-side culturing trials and tests by Corbin *et al.* (1998, 2000) at 20 versus 23°C demonstrated that the survival of *P. cornuta* was similar at either temperature, although animals grew larger and produced larger numbers of offspring at the warmer (i.e., 23°C) temperature. Control performance in 14-day *survival-and-growth* tests at 23°C was also superior to that at 20°C. It was concluded (Corbin *et al.*, 1998, 2000) that 23°C was the preferred temperature for culturing and testing this species.

While developing and standardizing this biological test method, the effect of different test chambers and feeding rations on the 14-day survival and growth of *P. cornuta* in control and test sediment was investigated by Environment Canada researchers. Consistent with other related sediment toxicity test methods (USEPA, 1990b; ASTM, 1994; USACE, 1995), toxicity tests with cultured *P. cornuta* were initially performed in 1-L glass jars or beakers with a 175-mL volume of sediment (~2-cm layer) and a 625-mL volume of overlying water (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000). Using this setup, a ration consisting of a 1:1 mixture of ground tropical fishfood flakes (e.g., TetraMarin™) and ground estuarine green algae (*Enteromorpha* sp.), fed three times a week at a rate of 5 mg dry weight per worm per feeding, was considered to be optimal for survival and growth without fouling problems due to excess food (Pocklington *et al.*, 1995). Subsequent studies demonstrated that test performance was simplified and improved using a 300-mL high-form glass beaker with a 50-mL volume of sediment and a 200-mL volume of overlying water, together with a reduced feeding ration (i.e., a 1:1 TetraMarin™:*Enteromorpha* mixture fed three times a week at a rate of 2 mg dry weight per worm per feeding) (Fennell, 1998; McPherson *et al.*, 1998). These improvements are incorporated in the biological test method described herein.

The sensitivity of *P. cornuta* to sediment grain size or organic enrichment has been investigated. Initial studies indicated some (marginal) reduction in 20-day survival rates for animals held in a field-collected "clean" reference sediment with 46% fines, relative to those for animals held in a negative control sediment with only 4% fines (Arenicola Marine, 1994; Pocklington *et al.*, 1995). Subsequent studies (Corbin *et al.*, 1998, 2000) found that, whereas the 14-day survival and growth of this species in 100% sand or 25% clay did not differ significantly from that in negative control sediment, growth and/or survival was reduced in artificial sediment with ≥50% clay as well as in field-collected reference sediment with 82% fines (65% silt and 17% clay). It was concluded from these studies that *P. cornuta* is sensitive to grain-size effects and, accordingly,

that field-collected reference sediment or artificial (formulated) *clean* sediment with similar grain-size characteristics to that of the test sediments need to be included in sediment toxicity tests with this species.

Studies by Corbin *et al.* (1998, 2000) demonstrated that the 14-day survival and growth of *P. cornuta* can be markedly reduced by artificially enriching sediment with organic material at concentrations as low as 1% total organic carbon, depending on the type of organic material added. Conversely, depending on the type of organic material, clean sediment artificially enriched with up to 5% total organic carbon can enable 14-day survival and growth of this species at rates similar to those in negative control sediment (Corbin *et al.*, 1998, 2000). Confounding toxic effects due to a high degree of organic enrichment of test sediments can be caused by variables associated with the degree of organic enrichment (e.g., elevated levels of ammonia and/or hydrogen sulphide in sediment pore water), which are not attributable to organic content *per se* (Corbin *et al.*, 1998, 2000).

The tolerance of *P. cornuta* to a range of concentrations of ammonia in sediment pore water has been investigated, using *artificial sediment* (100% silica sand) spiked with ammonium chloride. Results indicated a 14-day *LC50* for porewater ammonia of 21.7 mg N/L as total ammonia, and 0.3 mg N/L as un-ionized ammonia (Corbin *et al.*, 2000). Growth of *P. cornuta* was inhibited by porewater ammonia concentrations ≥3.9 mg N/L as total ammonia, or ≥0.05 mg N/L as un-ionized ammonia. Comparison of these findings with those for the spionid polychaete *B. proboscidea* (Corbin *et al.*, 2000) or the marine polychaete *Neanthes arenaceodentata* (Dillon *et al.*, 1993a; Bailey *et al.*, 1997) indicates that *P. cornuta* is more sensitive to elevated concentrations of ammonia in pore water than either of these two species of polychaete worms.

Four-day *water-only* reference toxicity tests with cadmium chloride, performed with cultured *P. cornuta* at 23°C, have revealed 96-h LC50s ranging from 3 to 9 mg Cd/L (McPherson *et al.*, 1998; Doe *et al.*, 2000a). In two series of comparative tests involving four laboratories, the overall mean LC50s were 5.4 and 5.2 mg Cd/L (SDs, 1.5 and 2.4 mg/L, respectively) (McPherson *et al.*, 1998). For this *reference toxicant* and test method at least, the sensitivity of cultured *P. cornuta* in *water-only* lethality tests appears to be somewhat greater than that for juvenile, cultured *N. arenaceodentata* (e.g., *water-only* 96-h LC50s, 12–14 mg Cd/L; Reish *et al.*, 1976; Reish, 1984; Reish and Gerlinger, 1997).

Comparative 14-day *survival-and-growth* tests with both *P. cornuta* and the spionid polychaete *B. proboscidea* were performed by each of four laboratories using a number of clean or contaminated field-collected sediments as well as a range of concentrations of copper-spiked sediment. Results from these tests demonstrated that *P. cornuta* was more sensitive to contaminant effects than *B. proboscidea* (McPherson *et al.*, 1998). Using *P. cornuta*, interlaboratory coefficients of variation (CVs) for grand means (all laboratories) of the 14-day survival data for each of the five concentrations of copper-spiked sediment in which some but not all worms survived, ranged from 4 to 46%. The grand-mean CVs for dry weight of *P. cornuta* in these concentrations at test end ranged from 28 to 53%, depending on concentration (McPherson *et al.*, 1998).

Associated tests by Farrar *et al.* (1998) included 14-day *survival-and-growth* tests with multiple concentrations of copper-spiked sediment using two species of spionid polychaete worms (*P. cornuta* and *B. proboscidea*; McPherson *et al.*, 1998), together with each of the following biological test methods and species:

- 28-day *survival-and-growth* tests using the marine polychaete worm *Neanthes arenaceodentata*;
- 14-day *survival-and-reproduction* tests using

the meiofaunal copepod *Schizopera knabenia*; and

- 28-day *survival, growth, and reproduction* tests using the estuarine amphipod *Leptocheirus plumulosus*.

Results for this series of tests indicated relative sensitivities to copper-spiked sediment as follows: *S. knabenia* > *N. arenaceodentata* > *P. cornuta* > *B. proboscidea* = *L. plumulosus* (Farrar *et al.*, 1998). Earlier *survival-and-growth* or *survival-only* tests for sediment toxicity using various species of marine or estuarine polychaete worms common to Canadian coastal waters, and identical test durations, have shown that *P. cornuta* is amongst the most sensitive of the test species examined in these studies (Arenicola Marine, 1993, 1994).

Test Organisms

2.1 Species and Life Stage

Laboratory-cultured spionid polychaete worms of the species *Polydora cornuta* are used in this biological test method. This species is a tube-dwelling, surface deposit feeder which normally resides within the upper layer (e.g., ≤2 cm) of estuarine or marine sediment. The identification, distribution, and life history of *P. cornuta* is summarized in Section 1.3. Appendix G should be consulted for further information regarding the identification and life history of this species; including aspects of its distribution, ecology, reproduction, and development. Species identification should be confirmed and documented by qualified personnel experienced in identifying this species of polychaete worm, using the distinguishing taxonomic features described and illustrated in Appendix G.

The sediment toxicity tests described in this report are to be started using newly settled and metamorphosed juveniles which have been cultured in the laboratory. Toxicity tests with *P. cornuta* should use juveniles that have been cultured at 23 ± 2°C and are three to four weeks post-release.[4] For greater standardization and reduced variability of dry-weight measurements it is desirable, although not always practical, to use animals that differ in age by only one or two days (see Section 2.3.10). The mean dry weight of the batch of juvenile worms used to start the test must range within 0.06 to 0.5 mg per individual worm (Doe *et al.*, 2000b; see Section 2.3.10).

2.2 Source and Acclimation

All worms used in a test *must* be derived from the same population. Sources of animals required to establish cultures (see Section 2.3) *may* be government or private laboratories which are culturing *P. cornuta* for sediment toxicity tests, or a commercial biological supplier.[5]

Breeding stock *can* be acquired from the following Canadian sources:

Toxicology Laboratory
Environmental Quality Section
Atlantic Environmental Science Centre
Environment Canada
P.O. Box 23005
Moncton, NB, E1A 6S8
Phone: (506) 851-3486

Environmental Toxicology Section
Pacific Environmental Science Centre
Environment Canada
2645 Dollarton Highway
North Vancouver, BC, V7H 1B1
Phone: (604) 924-2513

[4] The age of worms is referred to here and elsewhere in the report as the number of days "post-release". This represents the number of days following the release of developing larvae from the egg capsule within the adult female to the seawater beyond the adult. Distinguishing the age of worms as "x days post-release" rather than "x days old" is necessary, inasmuch as the time from fertilization until release of larvae to the surrounding medium is not known and cannot be determined using the present procedures of observation.

[5] Investigators might be concerned with the effects of excessive inbreeding of laboratory cultures, or might wish to use progeny reproduced from organisms that occupied a particular locale. Accordingly, cultures may also be established using wild populations. Guidance regarding suitable collection sites is given in Appendix G. If animals are obtained from a wild population, their taxonomy should be confirmed and they should be cultured through several generations and evaluated for sensitivity to reference toxicant(s) before the progeny are used in toxicity tests. Obtaining wild populations of organisms for testing should be avoided unless the ability of the wild population to cross-breed with existing laboratory populations has been demonstrated (USEPA, 1994a).

Breeding stock should be transported to the laboratory using the source of water in which the organisms have been reared. Water used for transporting animals should be well oxygenated (90 to 100% saturated) before shipment, and suitable substrate should be provided (see Section 2.3.7). Shipping containers should be insulated to minimize changes in water temperature during transit. Live organisms should be transported quickly to ensure their prompt (i.e., within 24 h) delivery. Excessive crowding of animals during shipment should be avoided to minimize stress and prevent oxygen deficiency in transit.

Upon arrival at the laboratory, organisms may be held in the water used in transit while temperature adjustments are made, or they may be transferred to well-oxygenated culture water adjusted to the temperature and salinity of the water in the shipping container. Gradual exposure of organisms to culture water is recommended in instances where there is a marked difference in quality (e.g., salinity, pH) from that to which they were previously acclimated. Guidance given in Section 4.1 for acclimating organisms to *test water might* also be followed here when transferring worms from another source to culture water.

Water temperature and salinity should be adjusted gradually to culture conditions (see Section 2.3), at rates not exceeding 2°C/day (USEPA, 1994a; EC, 1997a, b) and 5‰/day (EC, 1992a), respectively. During this *acclimation* period, seawater used to hold the breeding stock (see Sections 2.3.4 and 3.4) should be aerated gently. Other conditions during this interim holding period for acclimation of breeding stock to laboratory conditions should be as similar as possible to those used for maintaining cultures (Section 2.3).

2.3 Culturing

2.3.1 General

General guidance and recommendations for culturing *P. cornuta* in preparation for sediment toxicity tests are provided here. In keeping with the premise "*What might work well for one laboratory might not work as well for another laboratory*" (USEPA, 1994a; EC, 1997a, b), explicit directions regarding many aspects of culturing, including the choice of culture chamber, number of organisms per chamber, water-renewal conditions, substrate and food ration, are left to the discretion and experience of laboratory personnel; although guidance and recommendations are provided herein. Performance-based criteria[6] are used to evaluate the suitability of the cultured organisms for tests, and the acceptability of the test results. To be suitable for use in tests, cultures must have low mortalities, and the cultured organisms must appear healthy and behave and feed normally. Additionally, those used as controls in the test must have acceptably low mortality rates (Section 4.2). The acceptability of the culture should also be demonstrated by concurrent or ongoing tests using a reference toxicant (Section 4.9). If a *batch* or culture of organisms fails to meet these criteria, it should be discarded.

It is the responsibility of the laboratory to demonstrate its ability to obtain consistent, precise results using a reference toxicant, when initially setting up to perform sediment toxicity tests with cultured *P. cornuta*. For this purpose, intralaboratory *precision*, expressed as a *coefficient of variation* for the respective LC50 data, should be determined by performing five or more tests with different batches of test organisms from the same source, using the same reference toxicant (in this instance, cadmium chloride) and identical procedures and conditions for each test (see Section 4.9) (USEPA, 1994a; EC, 1997a, b). The laboratory should also confirm its test precision at this time by conducting five or more

[6] Performance-based criteria include those related to the survival and condition of cultured animals intended for use in the test (Section 2.3.11); as well as the criteria that must be met by control organisms for a test to be valid (Section 4.2), and those related to the performance of groups of animals in reference toxicity tests (Section 4.9).

14-day *survival-and-growth* tests using negative control sediment and different batches of test organisms (USEPA, 1994a; EC, 1997a, b). The conditions and procedures used to perform these initial tests with negative control sediment should be identical and according to Section 4.

When routinely performing sediment toxicity tests with *P. cornuta*, reference toxicity tests should be conducted monthly with the laboratory's cultures, using the conditions and procedures outlined in Section 4.9. If this monthly routine is not followed, the performance of individuals from the culture used to start a sediment toxicity test should be evaluated in a reference toxicity test conducted concurrently. Additionally, the performance of any cultures that have been recently established using new breeding stock (Section 2.2) should be checked with a reference toxicity test, and the results determined to be acceptable (see Sections 2.3.11 and 4.9) before these cultures are used to provide test organisms.

Cultures of *P. cornuta* should be frequently and routinely observed (e.g., daily or, as a minimum, 2–3 times per week on non-consecutive days). Ideally, records should be maintained which document the number of viable larvae used to start each culture, survival and weight of the larvae as they develop, estimated number of surviving adults and the production of young in each culture chamber, dates of culture renewals, numbers and age classes of transferred individuals, feeding regime (including type and quantity added on each occasion), together with water quality measurements.

A summary of the various conditions and procedures used by USEPA (1990b), ASTM (1994), and USACE (1995) for culturing polychaete worms is provided in Appendix D. These procedural specifics have presumably worked well in producing *Neanthes* sp. for use in sediment toxicity tests and, unless indicated otherwise in this report, provide useful guidance which may also be applied here. A checklist of recommended conditions and procedures for culturing *P. cornuta* to generate offspring for use in sediment toxicity tests is given in Table 1. Appendix H should be consulted for further details.

2.3.2 Facilities and Apparatus

Worms must be cultured in a controlled-temperature laboratory facility. Equipment for temperature control (i.e., incubator, recirculating water bath, or constant temperature room) must be adequate to maintain temperature within the required limits (Section 2.3.5). The culturing area must be isolated from any testing, sample storage, or sample-preparation areas, to avoid contamination from these sources. It must be designed and constructed to prevent contamination of cultures (e.g., elimination of copper or galvanized piping or fixtures that could drip metal-contaminated condensates). The air supply to this area should be designed and operated to prevent entry or recirculation of air from the testing facility or from other portions of the laboratory where contaminants are present.

All equipment, containers, and accessories that might contact the organisms or water within the culturing facility must be clean, rinsed as appropriate, and made of nontoxic materials (e.g., glass, Teflon™, type 316 stainless steel, nylon, Nalgene™, porcelain, polyethylene, polypropylene, fibreglass). Toxic materials including copper, zinc, brass, galvanized metal, lead, and natural rubber must not come in contact with this apparatus and equipment, or the culture water. Online compressed air to the culturing facility should be filtered as necessary to ensure that it is free of oil and fumes.

Various containers, such as covered Pyrex™ crystallizing dishes measuring 12.5 cm diameter × 6.5 cm deep, Corning™ casserole dishes, or 2- or 4-L plastic pails with lids, are suitable chambers for rearing cultures. The choice of container might be influenced by the number of juvenile worms required for a series of toxicity tests.

Table 1 Checklist of Recommended Conditions and Procedures for Culturing *Polydora cornuta* for Use in Sediment Toxicity Tests

Source of cultured worms	–	natural (intertidal sand-mud flats or subtidal), or from a culture maintained at another laboratory; all animals to be used in a test from the same source; species identification confirmed
Acclimation	–	gradually (≤2°C/day, ≤5 ‰/day) upon arrival or at any time thereafter, for temperature and/or salinity differences relative to culture or test water
Source and salinity of culture water	–	uncontaminated natural or reconstituted seawater; salinity, 30 ± 4‰
Water renewal	–	none (except replacements for evaporation) until larvae settle, with 80 to 100% renewal thereafter once per week
Monitoring water quality	–	temperature, salinity, dissolved oxygen, and pH measured at least weekly and during 24-h period preceding start of test
Temperature	–	23 ± 1°C as daily average, and 23 ± 3°C as instantaneous
Aeration/oxygen	–	aerate cultures gently; maintain dissolved oxygen at ≥80% saturation
Lighting	–	500 to 1000 lux adjacent to water surface; overhead full-spectrum tubes (fluorescent or equivalent, with a broad-spectrum wavelength); photoperiod 16-h light:8-h dark
Substrate	–	clean natural or formulated sediment (e.g., clean sediment from a site where field-collected specimens of *P. cornuta* are found)
Feeding	–	varies with life stage and number of individuals per culture; 1:1 mixture of ground TetraMarin™ and *Enteromorpha* sp., and/or the alga *Dunaliella tertiolecta* (see Appendix H for recommended quantities and rates)
Age/size for test	–	juveniles; 3 to 4 weeks' post-release; mean dry weight per worm must be within the range of 0.06 to 0.5 mg
Health criteria	–	discard adult worms in culture chamber if, at any time, >20% die or appear stressed

2.3.3 Lighting

Overhead broad-spectrum (fluorescent or equivalent) lights should illuminate the cultures. *Photoperiod* should be regulated at 16-h light and 8-h dark. Light intensity adjacent to the surface of the water in culture chambers should be within the range of 500 to 1000 lux.

2.3.4 Culture Water

Sources of water for culturing test organisms may be an uncontaminated supply of natural *estuarine* or *marine* water, or reconstituted seawater made up to a desired salinity according to Environment Canada's recommended procedure (EC, 2001b) (see Section 3.4). The salinity of the culture water

must be within the tolerance limits for *P. cornuta*; i.e., within the range of 15 to 35‰ (Section 1.4). Based on the (good) 14-day survival and *growth* of *P. cornuta* acclimated to a salinity of 30‰ and tested at salinities ranging from 10 to 30‰ (Corbin *et al.*, 2000), a culturing salinity of 30 ± 4‰ is recommended for all test salinities within this range.

Acceptable water must allow satisfactory survival, growth, and reproduction of *P. cornuta* under controlled laboratory conditions. The characteristics of the water used within a laboratory for culturing polychaete worms should be reasonably uniform, to improve the likelihood of culturing success and to minimize variations in the condition and development of cultured organisms.

The selection of an appropriate water source for use with cultures depends on a number of considerations, including the euryhalinity (i.e., range of salinities preferred and tolerated) of *P. cornuta* (see Section 1.4), the proven performance of this species in seawater supplies on hand or available to the laboratory, the range of porewater salinities in the test sediments, and the need to acclimate cultures to a particular salinity before their use in a test. Sections 3.4, 4.4, 5.4, and 6.3 should be consulted for guidance on the choice of water to be used for culturing worms in preparation for sediment toxicity tests. Guidance regarding an appraisal of the quality of the various prospective waters to be used for culturing (and testing) organisms, and for preparing reconstituted seawater or treating natural seawater (e.g., filtering, sterilizing, adjusting salinity, equilibrating for temperature and dissolved oxygen) before its use as culture (or test) water, is given in Section 3.4.

The quality of water in culture chambers should be monitored and recorded routinely. Water temperature, salinity, dissolved oxygen, and *pH* should be measured at least weekly, as well as during the 24-h period preceding the start of the test. Other water quality characteristics (e.g., nitrite, ammonia, hydrogen sulphide, suspended solids, total dissolved gases, metals, pesticides, and any other contaminants of concern) should be analyzed as frequently as is necessary to document water quality (e.g., quarterly). For each analytical method used, the detection limit should be appreciably (e.g., 3 to 10 times) below either (a) the concentration in the water, or (b) the lowest concentration that has been shown to adversely affect the survival, attained weight, or reproduction of polychaete worms or other sensitive marine or estuarine animals.

The water within the culture chambers should be aerated gently and continuously, and renewed routinely.[7] This may be accomplished manually, or automatically using suitable apparatus and techniques for continuous or *intermittent renewal*. A weekly exchange of 80 to 100% of the water in culture chambers is recommended, unless water is re-circulated through commercial (aquarium-supply) filters. Depending on the experimental setup used, including the density of animals in the culture, a greater rate of renewal might be necessary or prudent to assure healthy cultures.

2.3.5 Temperature

The temperature of the water in culture chambers containing *P. cornuta* must be 23 ± 1°C as a daily average. Additionally, the instantaneous temperature of the culture water must be 23 ± 3°C.

2.3.6 Dissolved Oxygen

Water to be used for cultures should be aerated vigorously just before use, to ensure adequate oxygen content and to prevent supersaturation with gases. Dissolved oxygen (DO) should be measured at this time to confirm that a satisfactory value has been obtained (e.g., 80 to 100% saturation).

The water in the culture chambers should be aerated gently (e.g., 2-3 bubbles/s for each litre of

[7] Continuous recirculation of culture water through commercial aquarium-supply filters can also maintain good-quality water within cultures. Such apparatus may be used as an alternative or supplement to water renewal (depending on the water quality maintained and on the performance of the cultures).

water) using filtered, oil-free compressed air. Air to the cultures should be dispensed through disposable airline tubing and disposable glass or plastic pipettes or, for large-volume cultures, aquarium-supply airstones. To ensure that dissolved oxygen is adequate to sustain optimum survival and growth of test organisms, it is recommended that DO in cultures be maintained at 80 to 100% saturation.

2.3.7 Culturing Substrate

Cultures of spionid worms have been successfully established (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000; Fennell, 1998; McPherson *et al.*, 1998; Jackman *et al.*, 1999) using sediment taken from a *site* where wild populations of *P. cornuta* exist. This field-collected sediment has also been used successfully as *negative control sediment* (Section 3.5) for 14-day *survival-and-growth* toxicity tests. Each sample of field-collected sediment used as culturing substrate or as negative control sediment in tests should be sieved (0.5 mm) to remove detritus and large indigenous organisms, then washed with culture water (Corbin *et al.*, 2000), and then frozen in small-volume (e.g., 500 mL) containers. When required, each small portion should be thawed, washed three times (Section 3.5), and used quickly to prevent its deterioration in quality and resulting problems (Corbin *et al.*, 2000). Success in establishing and maintaining healthy cultures might also be achieved using an artificial (laboratory formulated) *negative control sediment* (Section 3.5).

2.3.8 Food and Feeding

The diet and feeding rate for cultures of *P. cornuta* differ depending on the life stage. The preferred food type for planktonic larvae is the unicellular algal species *Dunaliella tertiolecta*, cultured in Erdschreiber medium. Once the larvae have settled and are developing as juveniles, this food type should be supplemented with increasing quantities of estuarine green algae (*Enteromorpha* sp.) and tropical fishfood flakes (e.g., TetraMarin™), which have been mixed in equal quantities and ground to a fine powder (Corbin *et al.*, 2000). Appendix H provides details on the preparation and storage of these food types, and on the recommended quantities to be fed cultures depending on their age, life stage(s), and numbers of individuals therein.

A 1:1 mixture of dried, ground TetraMarin™ fishfood flakes and dried, ground *Enteromorpha* sp. has proven suitable for culturing juvenile and adult spionid worms (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000; Fennell, 1998; McPherson *et al.*, 1998; Jackman *et al.*, 1999). Guidance on the preparation and storage of this food source is given in Section 4.5, with further details provided in Appendix H. The addition of this mixture to cultures two or three times per week (non-consecutive days), as a seawater slurry, enables good survival and growth of these life stages.

The marine alga *Dunaliella tertiolecta* is recommended as a food source for culturing the larval life stage of spionid worms (Reish, 1980b; Reish and Oshida, 1987; Pocklington *et al.*, 1995; D.J. Reish, personal communication, California State University, Long Beach, CA, 1995). This food source is also recommended as a supplemental diet for the juvenile and adult life stages of spionids (Corbin *et al.*, 2000). Feeding spionid worms two or three times per week on non-consecutive days (e.g., Mondays, Wednesdays, Fridays; or Tuesdays and Fridays) with a culture of *D. tertiolecta*, has been found to enable good growth and development of both *P. cornuta* and *B. proboscidea* (Pocklington *et al.*, 1995; Corbin *et al.*, 1998, 2000; Fennell, 1998; McPherson *et al.*, 1998; Jackman *et al.*, 1999). Appendix H should be consulted for detailed guidance on preparing cultures of *D. tertiolecta* as well as the quantities to be fed weekly to known-age cultures of *P. cornuta* being reared to provide test organisms.

2.3.9 Handling Organisms

The larval, juvenile, and adult life stages of *P. cornuta* should be handled as little as possible, to avoid damage and undue stress. When handling is necessary, it should be done gently, carefully, and quickly to minimize stress to the animals. Adult or younger individuals (juveniles or larvae) can be transferred between containers using a glass or

clear plastic pipette with a polished end and an opening of about 5 to 6 mm in diameter. Transferred organisms should be released below the water surface. Any animals that are dropped, injured, contact dry surfaces, or appear stressed when handled must not be used for testing.

Sieving of animals from sediment (e.g., for transfer to other culture containers or to test chambers) should be done as gently as possible, using a water bath rather than a stream of water to flush sediment through the screen. Organisms recovered by sieving should be kept submersed in culture or test water, and transferred as quickly as possible to other cultures or to test chambers (see Sections 2.3.10 and 4.1).

2.3.10 Juveniles for Toxicity Tests

The culturing procedures used for this biological test method must produce enough juvenile worms of a known and similar age (i.e., 3 to 4 weeks' post-release) and size to start the planned toxicity tests with sediment and/or reference toxicant. The mean dry weight of the group of worms used to start the test must be within the range of 0.06 to 0.5 mg per individual (Doe *et al.*, 2000b); and the variation in size between worms within the group should be minimized as much as possible. Additionally, the cultured organisms must meet specific performance criteria (see Sections 2.3.11, 4.2, and 4.9).

Cultures of *P. cornuta* should be maintained in the laboratory when toxicity tests with this species are required. The *mixed-age* culture(s) represent a mass culture of worms of various ages and life stages, maintained in one or more all-glass aquaria of an appropriate size. A 2- to 3-cm layer of clean sediment (Section 2.3.7) in the bottom of each aquarium provides a suitable substrate for the worms. The *known-age* cultures contain larvae or young juveniles that differ in age by no more than 7 days, and which are cultured in isolated dishes, pails, or other appropriate containers to provide test organisms that are 3 to 4 weeks' post-release in age (Corbin *et al.*, 2000). To be used as test organisms, these worms must have a mean dry weight per individual worm within the range of 0.06 to 0.5 mg (Doe *et al.*, 2000b).

Following, is a summary of procedures that might be used for generating *known-age* juvenile *P. cornuta* that are 3 to 4 weeks' post-release and of a suitable size (i.e., mean dry weight of 0.06 to 0.5 mg) for use in sediment toxicity tests. Further details are provided in Appendix H, as well as in Corbin *et al.* (2000). These procedures are recommended as some of several ways to obtain test organisms of a known and similar age and size. The number of organisms required for a particular test might influence the choice of procedure to be used, as might the similarity in age class desired. Laboratory personnel should familiarize themselves with these or other approaches, when gaining experience with culturing *P. cornuta* in preparation for toxicity tests.

To establish *known-age* cultures of *P. cornuta*, a group of 10 to 25 adult (broodstock) worms should be transferred from the *mixed-age* culture to each of several (e.g., 4 or 5) containers filled with culture water and containing a measured quantity of negative control sediment (Corbin *et al.*, 2000).[8] The containers for culturing broodstock should be aerated gently and continuously, and kept covered to reduce evaporation. The following actions should be taken two or three times a week (e.g., Monday, Wednesday, Friday; or Tuesday and Friday):

(i) Check each broodstock container for the presence of larvae. Determine larval abundance.

(ii) Using a transfer pipette, transfer newly released larvae to other culture chambers (e.g., 2-L or 4-L plastic pails) containing fresh culture water and negative control sediment.

[8] The choice of container for rearing broodstock adults or *known-age* test organisms depends on the number of *known-age* test organisms required for a particular study. Suitable containers include round Pyrex™ crystallizing dishes measuring 12.5 cm diameter × 6.5 cm deep and containing culture water plus 5 g of negative control sediment, or 2- to 4-litre plastic pails containing culture water plus 20 g of negative control sediment (Corbin *et al.*, 2000).

(iii) Pour off and immediately renew the culture water in all containers holding adult (broodstock) worms.

(iv) Add the appropriate food ration (see Section 2.3.8).

(v) Replace the lids and restart aeration.

All larval *P. cornuta* obtained over a several-day period (e.g., 3 to 7 days[9]) using this approach should be reared (following steps i, ii, iv, and v) as a pool of potential test organisms for a further three weeks, i.e. until the group is 3 to 4 weeks' post-release and has metamorphosed into juveniles ready for use in the test.

A narrower age range (e.g., ± 1 day) of organisms used to start a test might be desirable, especially since weight gain is measured as a primary test endpoint. One way to achieve this might be by rearing larvae released from adult female worms on the same day in segregated culture chambers containing a small amount (e.g., 20 g) of negative control sediment, until they are 3 to 4 weeks' post-release and all within ± 1 day in age. This approach, however, might prove impractical, depending on the number of test organisms required and on the number of "same age" larvae generated using this approach.

If sufficient numbers of larvae[10] for the planned tests are generated by adults held in broodstock culture chambers (as described previously) on a single day, this group of larvae could be transferred to a dish or pail containing culture water and a small amount of clean sediment (see Sections 2.3.7 and 3.5). Unless artificial sediment is used, the sediment should be previously frozen to remove indigenous organisms. Water in this culture chamber should be gently aerated (Section 2.3.6), and food should be provided.[11] After a holding period of 3 weeks, the organisms (now metamorphosed to young juveniles) in the tray or dish should be recovered by sorting the sediment in a white pan or tray and then transferring animals to test chambers using a wide-mouthed pipette and suitable transfer container (D.J. Reish, personal communication, California State University, Long Beach, CA, 1995), or by gently (Section 2.3.9) sieving it using a 0.5-mm mesh screen. If the latter technique is used, juvenile worms retained on the sieve should be rapidly[12] transferred to a sorting tray containing culture or test water (see Sections 2.3.4 and 3.4), and then transferred to test containers (Section 4.1).

The size of individual worms used to start the toxicity test should be as similar as possible. Sorting for similar size is recommended. This can be achieved by discarding those worms within the sorting tray that are obviously smaller or larger than the majority.

It is recommended that the average size of the worms within the *known-age* cultures be determined one or two days before the toxicity test is started, to make certain that they are within the required size range (Section 2.1). This can be achieved by measuring the dry weight of one or more samples of worms removed from the culture chamber(s) at this time. Alternatively, a random sample of ten or more "similar-sized" worms should be removed from the culture at this time, and their average body length determined using a dissecting microscope fitted with an ocular micrometer. Thereafter, an estimate of their mean dry weight can be attained using the linear regression of body length versus dry weight given in Doe *et al.* (2000b; Appendix 2).

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[9] This should be restricted to the minimal number of days (≤7 days) necessary to obtain an adequate number of *known-age* juveniles for the test(s) being planned, to ensure that age and size of organisms at the start of the test is as similar as possible.

[10] Using this procedure, it is desirable to obtain at least 3 times the number of larvae as is required for a particular test or series of tests, in order to recover enough *same-age* animals from the sediment for use in the test.

[11] Food should be added 2-3 times/week to the culture chamber. The type and quantity of food is age-dependent (see Appendix H).

[12] If this is not done rapidly, the young juveniles might crawl through the mesh of the sieve, making them difficult to recover.

2.3.11 Health Criteria

Cultures should be checked two or three times per week (e.g., Monday, Wednesday, and Friday; or Tuesday and Friday) as a minimum, and preferably daily. Records should be kept which monitor each culture's performance (see Sections 2.3.1, 2.3.4, 4.2, and 4.9). Procedures used to maintain each culture should be evaluated routinely, and adjusted as necessary to maintain or restore the health of the culture. Any adult worms seen emerged from their tubes should be prodded gently, and should be discarded if they appear to be dead, inactive, or unhealthy. If more than 20% of the adult broodstock worms in a culture chamber appear dead or inactive during any period of observation, the entire group in the container must be discarded.

Ideally, a reference toxicity test should be performed in conjunction with each sediment toxicity test. Laboratories routinely undertaking sediment toxicity tests using cultured *P. cornuta* may choose to conduct reference toxicity tests once each month, according to a regular schedule. All tests with reference toxicants should be performed using the conditions and procedures outlined in Section 4.9. Test-related criteria used to judge the health and sensitivity of the culture are given in Sections 4.2 and 4.9.

Biological measurements such as the average fecundity of mature females in a culture (e.g., average number of larvae released each week in each culture chamber), or the average time for animals to develop from eggs to sexually mature adults, might provide useful information on the health of the cultures. Ongoing records of these or other indices of the condition of cultures will likely prove useful, and are encouraged. No specific health criteria have been developed as yet with respect to physiological measurements, although they could be applied in the future.

Test System

3.1 Facilities and Apparatus

Tests may be performed in a water bath, environmental chamber, or equivalent facility having acceptable temperature and lighting (see Section 3.2) control. The test facility must maintain the daily mean temperature of all sediment and water in test chambers at 23 ± 1°C. The facility should be well ventilated to prevent exposure of personnel to harmful fumes, and isolated from physical disturbances or any contaminants that might affect the test organisms. The area used to manipulate sediment in preparation for tests should also be properly ventilated.

The test facility should be isolated from the area where worms are cultured, to avoid potential contamination of cultures. Additionally, the test facility should be removed from places where samples are stored or prepared, to prevent the possibility of contaminating the test chambers and the contents from these sources. The ventilation system should be designed, inspected, and operated to prevent air within the testing facility from contaminating culture facilities. Return air from sample handling and storage facilities or those where chemicals are processed or tested should not be circulated to the area of the laboratory where tests are conducted. Any construction materials that might contact the organisms, water, or test chambers within the facility must be nontoxic (see Section 2.3.2).

Compressed air used within the test facility for aerating water must be free of oil and fumes. Oil-free air pumps should be used wherever possible. Any oil or particulate in the air supply should be removed using inline filters, which are replaced as required to ensure their effectiveness.

Equipment and supplies that contact water, sediment, or test chambers should be chosen to minimize sorption of chemicals. Borosilicate glass, nylon, high-density polyethylene, high-density polystyrene, polycarbonate, fluorocarbon plastics, and type 316 stainless steel should be used whenever possible to minimize chemical sorption and leaching. The use of apparatus and supplies made of toxic substances (see Section 2.3.2) must be avoided.

Disposable glass or plastic pipettes and aquarium-supply airline tubing are required for delivery of a continuous (gentle) flow of air to each test chamber. Stainless steel (rather than brass) gang valves are recommended for regulating air flow. A supply of suitably sized watchglasses or lids is needed for covering individual test chambers.

The test facility must have the basic instruments required for monitoring the quality (e.g., temperature, pH, dissolved oxygen, salinity) of the test water and pore water. Additionally, the laboratory should be equipped to facilitate prompt and accurate analysis, with acceptable limits of detection, of unstable contaminants of potential concern such as ammonia, hydrogen sulphide, and (in instances where *dechlorinated municipal water* is used in the preparation of reconstituted or salinity-adjusted seawater) residual chlorine.

All test chambers, equipment, and supplies that might contact sediment or test water, must be clean and rinsed with test water, *deionized water*, or *distilled water*, before use. All nondisposable materials should be washed after use. The following cleaning procedure (USEPA, 1994a; EC, 1997a, b) is recommended, together with the useful cleaning guidelines provided in ASTM (1994).

1. Soak in tap water for 15 minutes, then scrub with detergent or clean in an automatic dishwater.

2. Rinse twice with tap water.

3. Rinse carefully with fresh, dilute (10%,

v:v[13]) nitric (HNO_3) or hydrochloric acid (HCl) to remove scale, metals, and bases.

4. Rinse twice with deionized water.

5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy). Use hexane for oily residues.

6. Rinse three times with high-quality deionized water.

Test chambers and apparatus that might contact sediment or test water should be thoroughly rinsed with test water, immediately before use in the test.

Before toxicity tests are undertaken in a new test facility, a minimum of five, 96-h *water-only* reference toxicity tests, and a minimum of five, 14-day *survival-and-growth* tests with negative control sediment, should be undertaken to confirm that acceptable performance of *P. cornuta* can be achieved using the new facility and the culturing and test conditions and procedures specified in this report (see Sections 2.3.1 and 4). Each test with reference toxicant or negative control sediment should be performed using a different batch of cultured organisms. Data from these preliminary tests should be compared by calculating and appraising the magnitude of the *coefficient of variation* for the respective series of tests and endpoint values.

3.2 Lighting

All test chambers should receive full-spectrum (e.g., fluorescent or equivalent) illumination from directly overhead, sufficient to provide 500 to 1000 *lux* adjacent to the surface of overlying water in test chambers. Illumination should be as uniform as possible for all test chambers. Photoperiod should be regulated at 16-h light and 8-h dark (EC, 1997a, b).

3.3 Test Chambers

High-form glass beakers with a capacity of 300 mL and an inner diameter of ~7 cm are to be used as test chambers (Fennell, 1998; McPherson *et al.*, 1998). Each beaker must be cleaned thoroughly before and after use (Section 3.1), and rinsed well with test water immediately before use. Each test chamber should have a clean glass (e.g., a suitably sized watch glass) or clear (transparent) plastic lid, to reduce the possibility of contamination of the contents and to minimize evaporation.

3.4 Test and Control/Dilution Water

Depending on the test design and intent (see Sections 5 and 6), *test water* (i.e., water overlying sediment in the test) and *control/dilution water* (i.e., water used to prepare dilutions of test chemicals and as control water in *water-only* exposures with a reference toxicant) may be either an uncontaminated supply of natural seawater, or reconstituted (artificial) seawater. The water supply used as test or control/dilution water is frequently the same as that used for culturing the test organisms (see Section 2.3.4), although it may be from another source. For instance, the use of *site* water, or *clean* water (natural or reconstituted) adjusted to the salinity of seawater at a collection site, might prove to be a good choice (see Sections 4.1, 4.4, and 5.4). Any seawater supply used routinely in the laboratory should be analyzed at least twice each year (ASTM, 1994) and as often as required to document its consistency in quality. Analyses of variables including salinity, pH, suspended solids, total organic carbon, chlorinated phenoxy herbicides, organophosphorus and organochlorine pesticides, polychlorinated biphenyls, ammonia nitrogen, nitrite, and metals are recommended.

It is extremely important that the test water and control/dilution water be uniform in quality and allow satisfactory survival and growth of the test organisms. Additionally, worms exposed to this water in control tests with sediment or reference toxicant should show no signs of disease, appear stressed, or exhibit atypical behaviour. The water source must, if possible (an exception might be if *site* water is used), have been previously demonstrated to allow acceptable survival and

[13] To prepare a 10% solution of acid, carefully add 10 mL of concentrated acid to 90 mL of deionized water.

growth of *P. cornuta* in 14-day tests with negative control sediment using the test conditions, procedures, and criteria for test validity given in Section 4, before being used in sediment toxicity tests with that organism.

The salinity of the test water must range within 10 to 35‰ (see Sections 1.4 and 4.4). It should be similar to the porewater salinity of the test sediment(s), and must be within 5‰ of that (Corbin *et al.*, 2000; see Section 4.4). If reconstituted (artificial) seawater is to be used, it should be made up to the desired salinity using hypersaline brine and/or dry ocean salts or reagent-grade salts, and the appropriate quantity of deionized or distilled water (see EC, 2001b for guidance). Any reconstituted water prepared by the direct addition of dry salts must be aerated vigorously for a minimum of 24 h before being used (EC, 2001b). Water added on any given day to each test chamber must be from the same *batch*; and the test water used to initiate the test and each renewal must be from the same source.

The use of hypersaline brine (*HSB*) derived from an uncontaminated source of natural seawater is recommended (EC, 2001b). Artificial hypersaline brine may also be prepared using commercially available dry ocean salts (e.g., Instant Ocean™) or reagent-grade salts (i.e., "modified GP2"; see USEPA, 1994b, Table 2 or USEPA, 1995). Any artificial HSB which is prepared using commercial sea salt or reagent-grade salts, however, must be filtered (≤1 µm) and then aerated vigorously for a minimum of 24 h before use (EC, 2001b). Reconstituted seawater should be filtered (≤5 µm) shortly before use to remove suspended particles, and should be used within 24 h of filtration (USEPA, 1994a).

Hypersaline brine may be prepared by concentrating seawater (natural or, less desirably, artificial) by freezing or evaporation. Once prepared, its salinity should be 90 ± 1 g/kg (EC, 2001b). If prepared by freezing, freeze at -10°C to -20°C for ≥6 h, and collect the HSB under the ice when it reaches a salinity of 90 ± 1 g/kg. If prepared by evaporation, heat the seawater in a non-corrosive, non-toxic container at ≤40°C while aerating it, until the desired salinity (i.e., 90 ± 1 g/kg) is achieved (USEPA, 1994b, 1995; EC, 2001b). Hypersaline brine may be added to natural seawater, distilled water, or deionized water, to increase the salinity to the level desired for testing. Guidance in Environment Canada (2001b) should be followed when preparing, aging, and storing HSB.

Depending on source and quality, natural or artificial seawater to be used for culturing *P. cornuta* and as test water should be filtered (≤ 5 µm) shortly before use, to remove suspended particles and organisms. Water that might be contaminated with pathogens should be treated shortly before use by further filtration (≤0.45 µm) and/or by ultraviolet sterilization (ASTM, 1994). If stored for more than one day, natural or reconstituted seawater should be held in clean, covered containers at 4 ± 2°C, and used within 14 days.[14] *Reconstituted water* should be homogeneous and aerated intensively before use. The solution should be filtered if turbidity, suspended solids, or a precipitate are evident.

Test and control/dilution water must be adjusted to the test temperature before use. The dissolved oxygen (DO) content of the water should be 90 to 100% of the air-saturation value at this temperature. As necessary, the required volume of water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and its dissolved oxygen content checked to confirm that 90 to 100% saturation has been achieved. The pH and salinity of this equilibrated (for temperature and DO) test water should be measured and stable before it is used in a test.

3.5 Negative Control Sediment

[14] USEPA (1994b) indicates that samples of natural seawater stored in clean, covered containers at 4°C should be used within 2 days. Such a brief storage period might not always be possible nor essential, although storage of natural or reconstitued (artificial) seawater should not exceed two weeks (i.e., 14 days).

Each sediment toxicity test must include an experimental control, with a minimum of five replicate beakers or jars containing *negative control sediment*. A negative control sediment is a sediment that is essentially free of any contaminants that could adversely affect the survival, growth, or behaviour of juvenile *P. cornuta* during the test. The use of negative control sediment provides a measure of test acceptability (i.e., mean survival at test end must be ≥90%), evidence of the health and behaviour of the test organisms, and a basis for interpreting data derived from the *test sediment*s.

Negative control sediment may be *clean* sediment taken from the same source as that used for maintaining the culture (Section 2.3.7), natural sediment taken from another collection site removed from known sources of contaminants and shown previously to enable acceptable survival and growth of the test species under the conditions of the test, or artificial sediment.[15] Any sediment showing evidence of contamination (e.g., an oil sheen) should be discarded. During the collection of natural sediment for use as negative control sediment, it is recommended that the sample's temperature and porewater salinity be measured and recorded, and that a composite sample derived from equal portions of all shovelfuls, dredge hauls, or grabs be taken and analyzed for its percent water content, particle size distribution, organic content, and concentration of any organic or inorganic contaminants.

The selection of an appropriate negative control sediment depends on considerations such as the study design, physicochemical characteristics of the test sediment(s), and the availability of suitable *clean* sediment having the desired properties. There should also be evidence that the sediment provides consistent and acceptable biological endpoints using *P. cornuta* and this biological test method. While many clean, *natural* sediments have been used as negative control sediment in growth and/or survival toxicity tests with marine or estuarine polychaete worms, the use of *artificial* negative control sediment offers a more consistent, standardized approach and one which numerous researchers are now actively pursuing (e.g., Suedel and Rodgers, 1994; USEPA, 1994a; Suedel *et al.*, 1996). At present, it is premature to recommend a recipe for *artificial* negative control sediment which is suitable for a 14-day *survival-and-growth* test using *P. cornuta*.

The investigator should be aware that the presence of indigenous macro-organisms in samples of negative control sediment (or in reference or test sediment) can in some instances reduce the growth of juvenile polychaetes or other infaunal invertebrates used in sediment toxicity tests, and that this can confound the interpretation of test results (Reynoldson *et al.*, 1994). Additionally, samples of field-collected sediment intended for use as negative control sediment might contain larval or juvenile spionids or perhaps other species of small infaunal polychaetes which could be misconstrued as test organisms at the end of the test. These problems are prevented by freezing negative control sediment before its use in the test.

Unlike samples of *reference sediment* or *test sediment*, which must not be wet-sieved or frozen (see Section 5.3), samples of field-collected *negative control sediment* should be washed through a fine-mesh (e.g., 0.5 mm) sieve to remove indigenous macro-organisms and, if necessary, to adjust the salinity of the interstitial water to that desired for the test. Water used for sieving should be clean test water (Section 3.4). If necessary, negative control sediment can be

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[15] Artificial negative control sediment is also described as reconstituted, formulated, or synthetic sediment. It is typically prepared using sand, silt, clay, and nontoxic organic constituents obtained from commercial sources, and hydrated with reconstituted or natural seawater. Artificial sediment can be prepared to match different natural sediments with respect to particle size distribution, organic carbon content, salinity, pH, cation exchange capacity, etc. (Suedel and Rodgers, 1994). Alternatively, one or more recipes can be used to prepare standardized *negative control sediment*(s) for routine use in sediment toxicity tests with *P. cornuta*.

sieved twice (once to remove small indigenous macroorganisms and once to adjust its porewater salinity to a specific value; which is usually that of the overlying water introduced to each test chamber). After each sieving, the sediment and water used for sieving should be left together, undisturbed, for a period of time (e.g., overnight) sufficient to allow the settling of fine particles suspended in the sieve water. The overlying water should then be decanted and discarded, and the sediment mixed to re-distribute the fine particles. After sieving field-collected negative control sediment, using test water at the appropriate salinity, it is recommended that numerous small aliquots (e.g., ~300 mL volumes) be placed in suitable small plastic containers (e.g., 500-mL freezer bags), which are then sealed to exclude any air space and stored in the freezer at -20°C. When required, a suitable number of frozen aliquots of negative control sediment should be thawed and washed three times, while minimizing the loss of fines (Corbin *et al.*, 2000), to ensure the removal of soluble toxic constituents (e.g., ammonia and/or hydrogen sulphide). These washed negative control sediments should be used in cultures or sediment toxicity tests immediately thereafter.

3.6 Positive Control Sediment

The use of one or more samples of *positive control sediment* is recommended for inclusion in each series of 14-day *survival-and-growth* tests for sediment toxicity using *P. cornuta*, to assist in interpreting the test results. The *positive control sediment* might be a *standard contaminated sediment* such as one available through the National Research Council of Canada's Marine Analytical Chemistry Standard Program, Ottawa, ON. A second approach is to use a sample of *clean sediment* (*artificial sediment* or field-collected *reference sediment*) that has been spiked experimentally (Section 6) with one or more toxic chemicals or chemical products at a concentration toxic to *P. cornuta* according to this biological test method. A third option is to use a highly contaminated sample of field-collected sediment shown previously to be toxic to *P. cornuta* according to this biological test method. This option is not recommended, however, unless the characteristics (including performance in a 14-day *survival-and-growth* test using *P. cornuta*) of this sediment are well known beforehand. *Positive control sediment* may be used as a reference toxicant (see Section 4.9) when appraising the sensitivity of the test organisms and the precision and reliability of results obtained by the laboratory for that material.

3.7 Reference Sediment

One or more samples of *reference sediment* might be included in a 14-day *survival-and-growth* test for sediment toxicity using *P. cornuta*.[16] *Reference sediment* is field-collected sediment taken from a presumably *clean* (uncontaminated) site where the physicochemical properties (e.g., percent organic content, particle size distribution, and pH) represent the sample(s) of *test* (contaminated) *sediment* as much as possible. Ideally, the reference sediment is collected from the general vicinity of the site(s) where samples of test sediment are collected, but is removed from the source(s) of contamination.

3.8 Test Sediment

This biological test method is intended for use in measuring the toxicity of one or more samples or mixtures of contaminated or potentially contaminated sediment (*test sediment*), using *P. cornuta* as test organisms and their 14-day survival and growth as the biological endpoints. The sample(s) of test sediment might be either field-collected sediment from an industrial or other site of concern, or industrial or municipal sludge (or other dredged material) under consideration for possible ocean disposal. A sample of field-collected *test sediment* might be tested at a single concentration (typically, 100%) or evaluated for toxicity in a multi-concentration test whereby a series of concentrations are

[16] The use of field-collected *reference sediment* might not be appropriate for certain toxicity tests such as those using samples of sludge (Section 5) or chemical-spiked sediment (Section 6).

prepared by mixing measured quantities with either *negative control sediment* or *reference sediment* (see Section 5). The test sediment might also be one or more concentrations of a chemical-spiked sediment, prepared in the laboratory by mixing or adding one or more chemicals or chemical products with either *negative control sediment* or *reference sediment* (see Section 6).

Universal Test Procedures

General procedures and conditions for this biological test method apply when testing the toxicity of samples of sediment, particulate waste (e.g., sludge), or chemical, and to the associated reference toxicity tests. More specific procedures for conducting tests with field-collected samples of sediment or other similar particulate material (e.g., sludge, dewatered mine tailings, drilling mud residue) are provided in Section 5. Guidance and specific procedures for conducting tests with negative control sediment or other sediment spiked experimentally with chemical(s) or chemical product(s) are given in Section 6.

All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 2 describes not only universal procedures but also those for testing specific types of test substances or materials.

Universal procedures for performing a chronic sediment toxicity test are described herein. This test method uses the juvenile life stage of cultured spionid polychaete worms (i.e., *P. cornuta*) as test organisms, and measures survival and weight attained as biological endpoints. Test duration is 14 days. Animals are fed a measured and standard ration three times per week (non-consecutive days), the water overlying sediment in each test container is aerated gently and continuously, and the overlying water is renewed after seven days.

4.1 Beginning the Test

Each test chamber (see Section 3.3) placed within the test facility must be clearly coded or labelled to enable identification of the sample or its concentration. The date and time when the test is started must be recorded, either directly on the labels or on separate data sheets dedicated to the test. The test chambers should be positioned to allow observations and measurements to be made easily. *Treatment*s should be positioned randomly within the test facility (USEPA, 1994a; EC, 1997a, b).

The day that animals are initially exposed to samples of test materials or substances is designated Day 0. On the day preceding the start of the test (i.e., Day -1), each sample or subsample of test sediment or similar particulate material, including negative control sediment and *reference sediment*, should be mixed thoroughly[17] (see Sections 5.3 and 6.2) to provide a homogeneous mixture consistent in colour, texture, and water content. *Quantitative* measures of homogeneity might include particle size analysis, total organic carbon, percent water, and concentration of specific chemicals.

Immediately following mixing, replicate 50-mL volumes of the sample should be transferred to the test chambers. A minimum of five laboratory *replicate*s per treatment must be established (USEPA, 1990b; ASTM, 1994; USACE, 1995; EC, 1997a, b). The sediment added to each chamber should be smoothed using a spatula or by tapping the chamber against the side of the hand. Test water (see Section 3.4) is then poured slowly down the side of the beaker. To minimize the disruption of sediment as test water is added, a disc made of Teflon™, polyethylene, or nylon sheeting, cut to fit the inside diameter of the test chamber, may be placed on the sediment surface before water is added[18] (EC, 1992a, 1997a, 1997b;

[17] Any liquid that has separated from the sample during transport and/or storage must be remixed within the sample.

[18] A length of nylon monofilament line (or nontoxic equivalent) could be attached to the disc, to enable its removal once the test water is added. Alternatively, the disc could be cut from a polyethylene bag in a keyhole configuration which provides a circle with an attached portion for removal. The disc should be rinsed with test water if reused to prepare replicates of a treatment. A separate disc should be used for each treatment.

Table 2 Checklist of Recommended Conditions and Procedures for Conducting Sediment Toxicity Tests Using *Polydora cornuta*

Universal

Test type	–	sediment toxicity test for effects on survival and growth, with renewal of overlying water on Day 7 only
Test duration	–	14 days
Water replacement	–	renewal of overlying water in each test chamber on Day 7, by siphoning out ~80% of volume and renewing it with fresh seawater adjusted to test temperature and salinity
Test (overlying) water	–	culture water or other clean seawater, natural or reconstituted; salinity must be within the range 10 to 35‰ and within 5‰ of the porewater salinity of test sediment(s); adjusted to test temperature before use; dissolved oxygen, 90 to 100% saturation
Negative control sediment	–	sample of clean sediment that is used routinely to assess the performance of the test organisms and the acceptability of the test; either natural or artificial sediment
Test organisms	–	cultured juveniles; 3 to 4 weeks post-release; mean dry weight of individual worms must be within the range of 0.06 to 0.5 mg at start of test; normally 5 worms per test chamber
Test chamber	–	300-mL high form glass beaker covered with lid (e.g., watchglass); internal diameter, ~7 cm
Volume and depth of wet sediment	–	50 mL, ~2-cm layer
Volume of overlying water	–	200 mL (made up to 250-mL mark on test chamber)
Number of replicates	–	recommend ≥5 field replicates, each a discrete (i.e., different) sample from the same location; must be ≥5 laboratory replicates for each field replicate; must be ≥5 replicates per concentration (treatment) if a multi-concentration test with contaminant-spiked sediment is performed for regulatory purposes
Temperature	–	daily average, 23 ± 1°C; instantaneous, 23 ± 3°C
Lighting	–	overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark
Aeration	–	aerate overlying water in each test chamber overnight before start of test, as well as during test; aeration rate, continuous and minimal (e.g., 2 to 3 bubbles/s, each chamber) to maintain ≥90% saturation
Feeding	–	1:1 mixture of ground tropical fishfood flakes (e.g., TetraMarin™) and ground estuarine green algae (*Enteromorpha* sp.); 2 mg dry weight per worm each feeding; fed as seawater-wetted suspension, three times per week
Observations	–	optional: numbers of organisms in each chamber seen on sediment surface, and their behaviour (daily or less frequently)

Measurements of overlying water	–	temperature, ≥3 times/week; DO, salinity, pH, and ammonia, at start and end and just before renewal; measured in at least one test chamber representing each treatment
Endpoints	–	significantly lower survival and weight than in control or reference treatments (based on mean percent survival and mean dry weight, each treatment); 14-d LC50 for multi-concentration test, where appropriate; *ICp* for weight, where appropriate
Test validity	–	invalid if mean 14-day survival in negative control sediment <90%

Field-Collected Sediment or Similar Particulate Material

Transport and storage	–	if sample >7°C, cool to 7°C (ice or frozen gel packs); transport in dark at 1 to 7°C (preferably 4 ± 2°C); store in dark at 4 ± 2°C; test should start within two weeks and must start within six weeks
Reference sediment	–	one or more samples for tests with field-collected sediment; taken from sites presumed to be clean but in the general vicinity of sites where test sediments are collected (i.e., same body of water); frequently selected for use in the toxicity test because of its physicochemical similarity (e.g., particle size and/or organic carbon content) to the test sediments
Sample characterization	–	at least particle size analysis (percent sand, silt, and clay), total organic carbon, percent water, porewater salinity, porewater pH, and porewater ammonia
Preparation of sample	–	only if necessary, remove debris and indigenous macro-organisms using forceps or a gloved hand, or by pressing (without using any water) the sample through a coarse (e.g., 5 mm) sieve; homogenize thoroughly by mixing by hand or using a rolling mill

Sediment Spiked with Chemical(s) or Chemical Product(s)

Characterization of chemical(s) added	–	information on stability, water solubility, vapour pressure, purity, and biodegradability should be known for chemical(s) spiked into negative control (or other) sediment
Solvent	–	test water is the preferred solvent; if an organic solvent is used, the test must include a solvent control
Preparation of mixtures	–	procedure depends on test design and objectives; might include one or more chemical concentrations mixed in control or test sediment, or specific chemical concentrations added to the test water overlying negative control (or other) sediment; chemical/sediment mixtures may be prepared manually or by mechanical agitation as slurries
Concentration of chemical(s) added	–	normally measure at beginning and end of test, in high, medium, and low strengths as a minimum
Test and dilution water	–	reconstituted water if a high degree of standardization is required

ASTM, 1994). A total volume of 150 mL of test water (or, depending on the test, a test solution) should be added at this time to the test chamber to bring the level to the 200-mL mark inscribed on the beaker's side.

The overlying water in each test chamber should be aerated overnight before the test organisms are introduced, and for the duration of the test (see Section 4.3). Each beaker should be kept covered (watchglass or clear plastic lid) during the pretest and test periods, to minimize evaporation and to reduce the possibility of contamination. If water other than culture water is used as the test water (see Sections 5.4 and 6.3), acclimation of test organisms to this water before the start of the test might be advisable or necessary to minimize any stress on the animals caused by different water quality characteristics.

Test organisms used to begin the test are those that are 3 to 4 weeks post-release on the day the test is initiated (see Section 2.3). The mean dry weight of individual worms must be within the range of 0.06 to 0.5 mg at this time.[19]

The porewater salinity of each test sediment or similar particulate material must be measured before the the test is initiated (USEPA, 1994b). For each sample, its porewater salinity must range within 10 to 35‰ if the sample is to be tested for toxicity using *P. cornuta* (see Sections 1.4 and 4.4) (Corbin *et al.*, 2000).

To start a test (i.e., on Day 0), portions of the negative control sediment within the *known-age* culture chambers (Section 2.3.10) should be sieved gently and quickly in either water within these chambers or using temperature- and DO-equilibrated test water (Section 3.4). Worms remaining on the sieve should be transferred rapidly to a shallow, white sorting tray containing equilibrated test water, until approximately one-third to one-half more than the number of worms required for the test have been retrieved. Any worms remaining in their mucoid tube should be probed gently from the tube using a fine-tipped artist's paint brush (e.g., camel hair), to observe their vitality and appearance. All worms in the sorting tray should then be examined closely under a dissecting microscope, and any appearing atypical in size, injured, discoloured, or otherwise unusual or mistreated, should be discarded. Remaining worms should represent those considered most suitable for the test (i.e., worms appearing active, apparently healthy, and of a similar size). Thereafter, five worms should be assigned and transferred randomly to each test chamber. These organisms should be handled as little and as carefully as possible (see Section 2.3.9) during their retrieval from *known-age* culture chambers and their subsequent transfer to the test chambers.

Individual worms to be transferred should be selected impartially, and sequentially pipetted carefully and gently into a series of small beakers, cups, or dishes, each containing approximately 25 mL of equilibrated test water, until each of these chambers contains five animals (ASTM, 1994). The number of worms in each transfer chamber should be verified by recounting them. The aeration to the test chambers should be stopped just before adding the worms. Each group of worms should then be added to a test chamber, by gently pouring the contents of the transfer chamber into the test vessel. Any animals remaining in the transfer beaker, cup, or dish should be washed (using equilibrated test water) gently and quickly into the test chamber. Following all transfers, the water level in each test chamber should be raised to the 250-mL mark inscribed on the beaker's side, using equilibrated

[19] Smaller animals are too small to find and handle, and larger ones are approaching sexual maturity and might therefore have lower growth rates during the test. Studies performed with differing size classes of test organisms at Environment Canada's Atlantic Environmental Science Centre (Doe *et al.*, 2000b) determined that the sensitivity of worms within this size range to contaminated sediment, as measured using a series of multi-concentration tests with a *standard contaminated sediment*, was unaffected by worm size based on the (similar) 14-day IC25 and LC50 values determined for each size class.

test water. The covers should be replaced on each test chamber, and the worms left undisturbed until they settle to the sediment. Thereafter, aeration of the overlying water should be restarted, and aeration rates checked and readjusted as necessary (Section 4.3).

During the transfer process, nine transfer chambers, each containing five test organisms, should be selected randomly to determine the initial dry weight of the group of animals used in the test. For each of three sets of three transfer chambers, randomly chosen from these nine, the contents of each (i.e., five test organisms) should be rinsed briefly (see Section 4.7) and placed together (n = 15) in a single pre-weighed weighing pan. Thereafter, the three weighing pans, each containing 15 worms, should be dried and weighed to determine the mean (± SD) initial dry weight of the test organisms on Day 0. The procedure for determining the initial dry weight of each replicate group (and the mean dry weight per individual worm) should be the same as that described for measuring dry weights of worms at the end of the test (see Section 4.7).

4.2 Test Conditions

- This is a 14-day sediment toxicity test, during which the overlying water must be renewed on Day 7 only.

- The test chamber is a 300-mL high form glass beaker, with an inner diameter of ~7 cm. Each container is covered with a watchglass or clear plastic lid.

- Control and test sediments must be present as a uniform, 50-mL layer approximately 2 cm in thickness, with a 200-mL volume of overlying water.

- The test must be conducted at a daily mean temperature (overlying water) of 23 ± 1°C. Additionally, the instantaneous temperature must always be 23 ± 3°C.

- Test chambers must be illuminated with a daily photoperiod of 16-h light and 8-h dark, using overhead, full-spectrum lights (fluorescent or equivalent). Light intensity adjacent to the surface of the overlying water should be 500 to 1000 lux.

- The overlying water in each chamber should be aerated continuously at a minimal rate (see Section 4.3).

- Organisms in each test chamber must be fed three times per week (see Section 4.5).

- For a valid test, the mean survival rate for animals in negative control sediment must be ≥90% at the end of the test.

4.3 Dissolved Oxygen and Aeration

The overlying water in each test chamber should be aerated continuously on the night preceding the start of the test (see Section 4.1), as well as during the test. Compressed air, filtered if necessary so as to be free of oil, should be bubbled through a disposable glass pipette. The tip of the pipette should be suspended approximately 3 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle and regulated. The bubble rate within each test chamber (e.g., 2 to 3 bubbles/s) should be adequate to maintain a DO concentration in the overlying water that is ≥90% of saturation; and must not disturb the surface of the test sediment (EC, 1997a, b). Stainless steel gang valves are useful for regulating air flow, and do not pose a potential toxicity problem. The rate of air flow to each test chamber should be observed routinely (e.g., daily) throughout the test, and adjustments made if necessary to maintain a gentle rate of aeration. Aeration must not disturb the surface of the sediment.

4.4 Salinity and Renewal of Overlying Water

The salinity of the overlying water used in a test must be within the tolerance limits of juvenile *P.*

cornuta, i.e., it must range within 10 to 35‰ (see Section 1.4). The salinity of the test/overlying water must be within 5‰ of that of the pore water of the test sediment(s), when added to test chambers (Corbin *et al.*, 2000; Section 3.4). To achieve this, the salinity of the pore water in each sample of test (contaminated) sediment must be measured before the test. The salinity of the overlying water to be used in a test should be adjusted to the mean porewater salinity of one or more test sediments from a particular study area (EC, 1998). In the (unusual) instance where the porewater salinity of any of these test sediments differs by more than 5‰ from the mean value, the overlying water will need to be adjusted to and tested at differing salinities (two or more), to ensure that the salinity of the overlying water added to each test chamber is within 5‰ of that of the pore water of the test sediment therein.

For a given test, the salinity of each *batch* of test water used to initially make up the overlying water and renew it on Day 7 should not differ by more than 2‰. Water added on any given day to each test chamber must be from the same batch; and the test water used to initiate the test and for the Day-7 renewal must be from the same source. Section 3.4 provides guidance on preparing seawater of a specified salinity for use as test water.

Using an experimental design similar to that recommended here, weekly renewal of the overlying seawater has been previously demonstrated to maintain good water quality while minimizing the loss of contaminants leached from test sediments (USEPA, 1990a; USACE, 1995). On Day 7 of the test, the cover and air supply for each test chamber should be removed. Thereafter, approximately 80% of the overlying water in each test container should be renewed by siphoning and prompt replacement using test water previously equilibrated for temperature and dissolved oxygen (Section 3.4). Care should be taken during each siphoning and renewal, to minimize any disturbance of the test sediment. Good renewal techniques include the careful positioning of the siphon, continual observation of overlying water and sediment while siphoning, and the addition of renewal water by gently pouring it down the inside wall of the test chamber and by disbursing its force over the surface of a disc held temporarily above the surface of the sediment (Section 4.1).

4.5 Food and Feeding

Worms in each test chamber must be fed three times per week (on non-consecutive days, e.g., Monday, Wednesday, and Friday), on the day that the test is started and throughout the test period. Any feedings coincident with the day that the overlying water is renewed (Section 4.4) should be carried out following water renewal, to minimize any loss of food during this procedure.

Since dry weight of worms is a primary endpoint for the test, an identical food ration must be added to each test chamber on each feeding occasion. The ration provided must be adequate to enable acceptable survival and growth of the animals during the test period, but not be excessive. The addition of excess food or a ration different than that recommended here is to be avoided since it might alter the bioavailability of contaminants in the sediment, promote the growth of fungi or bacteria on the sediment surface, or otherwise reduce the standardization of the assay.

Food for test organisms should be prepared using a 1:1 mixture of ground tropical fishfood flakes (e.g., TetraMarin™)[20] and ground estuarine green algae (*Enteromorpha* sp.)[21]. Equal quantities of these two food sources should be mixed

[20] This and other commercial fishfood flakes are available from most tropical-fish supply houses.

[21] Enteromorpha is collected from estuarine mud flats or tidal pools, blotted dry, spread as a thin layer on a drying tray, and then oven-dried (e.g., 50°C for ≥24 h) (Reish and Oshida, 1987). Once it is completely dry, it is coarsely ground. A 1:1 mixture of dried *Enteromorpha* and TetraMarin™ is prepared by mixing equal quantities (e.g., 5 g) of each and grinding the dry mixture to a fine powder using a domestic food blender. The powder is stored in an air-tight container.

thoroughly. The dry food mixture may be stored refrigerated (4 ± 2°C) in a sealed container until required. At the time of each feeding, the quantity of this dry food mixture required for feeding all replicates (plus a surplus of ~25%) should be weighed and moistened with test water in a suitable beaker or cup. This mixture should then be ground thoroughly using a mortar and pestle, and a wetted slurry containing 25 mg dry food/mL test water prepared. Animals in each test chamber are to be fed an inoculum of 0.4 mL (equivalent to ~10 mg dry food) of the slurry on each feeding occasion. The slurry should be stirred continuously while pipetting the 1:1 food mixture to each test chamber, to ensure homogeneity of the suspension. This food ration is maintained throughout the exposure period, even though mortalities might occur during the test (USEPA, 1990b; ASTM, 1994; USACE, 1995).

4.6 Observations and Measurements During the Test

Detailed records of the food type and ration added to each test chamber should be made on each feeding occasion. Observations of the appearance of the sediment surface in each test chamber (i.e., any evidence of a fungal or bacterial growth) should be recorded at this time. Other observations of abnormal and/or unanticipated events (e.g., worms seen emerged from the sediment; air flow interrupted or excessive; turbidity in overlying water) should also be recorded during each period of observation.

Depending on study objectives or the clarity of overlying water in test chambers, it might be worthwhile to regularly (preferably, daily) check each test chamber, to observe and record the number of worms seen on the surface of the sediment. The investigator should be aware that such observations, which are intended to indicate avoidance responses of test organisms, are sometimes difficult or impossible to undertake due to turbidity of the overlying water.

The temperature of the overlying water must be measured at the beginning of the test, and thereafter at least three times per week on non-consecutive days (e.g., Mondays, Wednesdays, Fridays) until test completion. These measurements must be made in at least one test chamber representing each treatment; and more frequent (i.e., daily) measurements are recommended. Additionally, it is recommended that the temperature of any water bath used, and/or of the air in a temperature-controlled room or chamber used for the test, be recorded continuously.

For at least one test chamber representing each treatment, the concentration of dissolved oxygen in the overlying water must be measured at the beginning of the test, as well as just before its renewal and at the end of the test. More frequent (e.g., daily or three times per week on non-consecutive days) measurements are recommended and should be performed for sediments having a high oxygen demand. A probe and calibrated dissolved oxygen (DO) meter is recommended for these measurements. To minimize cross-contamination, the probe must be rinsed in deionized or distilled water between samples. The position of the tip of the pipette in each test chamber and the rate of aeration (Section 4.3) should be checked frequently and routinely throughout the test, and adjustments made as necessary.

If at any time during the test the air flow to one or more test chambers is observed to have stopped, the dissolved oxygen concentration in the overlying water must be measured and then the air flow reestablished at a gentle rate. Any DO readings which are noted to have fallen below 60% saturation (ASTM, 1994) must be included in the test-specific report (Section 7.1), and considered when interpreting the results.
In at least one test chamber representing each treatment, values for salinity and pH in the overlying water must be measured at the beginning of the test, as well as just before water

renewal and at the end of the test. Salinity and pH may be measured using probes and calibrated meters. As with DO measurements, any probe inserted in a test chamber must be rinsed in deionized or distilled water between samples.

Concentrations of ammonia in the overlying water must be measured (total ammonia; see for example APHA *et al.*, 1998) in at least one test chamber representing each treatment, at the beginning (i.e., just before the addition of test organisms) and end of the test as well as just before water renewal on Day 7. Total ammonia may be measured using an ion-specific electrode or by extracting an aliquot of the overlying water for this analysis. As with DO measurements, any probe inserted in a test chamber must be inspected carefully immediately after each reading, and rinsed in deionized or distilled water between samples. For measurements of ammonia requiring sample aliquots, a pipette should be used to carefully remove the required volume of water, which should be taken from a depth of about 1 to 2 cm above the sediment surface. On each occasion, no more than 10% of the volume of the overlying water in a test chamber should be removed for this purpose. For each measurement, the concentration of un-ionized ammonia should be calculated based on the concurrent measurements of pH and salinity of the overlying water and in consideration of test temperature (Trussell, 1972; Bower and Bidwell, 1978; USEPA, 1985a).

4.7 Ending a Test

The test is terminated after 14 days. Just before sieving the contents of a test chamber, all live and apparently dead worms observed on the surface of the sediment should be pipetted from the test chamber. Individuals which are completely inactive but not obviously dead (e.g., not decomposing) should be held in test water within a petri dish or other suitable container, and examined closely at this time using a low-power microscope or handheld magnifying glass. These individuals should be prodded gently with a sharp point to confirm that they show no sign of life (such as a parapodial twitch), and are then to be counted as dead.

Numbers of dead and surviving worms recovered by pipetting should be recorded. Dead animals should be discarded. All live animals should be placed in a numbered weighing pan or similarly small holding receptacle. This receptacle should contain a volume of test water sufficient for rinsing the worms and holding them briefly until all of the survivors sieved from the sediment in the test chamber are added to and rinsed in it.

A consistent amount of time should be taken to sieve and examine the contents of each test chamber closely for recovery of live or dead organisms. To ensure that the procedure used to recover worms is adequate, it is recommended that the laboratory personnel responsible for sieving the contents of test chambers previously demonstrate that they are able to retrieve an average of at least 90% of similar-sized animals from the sediment[22] (USEPA, 1994a; EC, 1997a, b).

The contents of each test chamber should be sieved through a 0.5-mm screen (USEPA, 1990b; ASTM, 1994; Arenicola Marine, 1994; Pocklington *et al.*, 1995) or, if preferred, through a series of stacked screens (2.0, 1.0, and 0.5 mm mesh size; USACE, 1995). The sieve should be shaken in a basin containing test water, rather than being sprayed with water to remove the sediment (USEPA, 1990b). Material retained on the screen(s) should be washed gently into a white sorting tray containing test water, and examined closely for live and apparently dead worms. Any worms remaining in tubes should be prodded gently to force them out; unresponsive animals

[22] USEPA (1994a) recommends a check on the recovery capability used by Tomasovic *et al.* (1995), whereby test organisms are added to negative control sediment and their recovery determined after 1 h using the same technique as that used for sieving the contents of test chambers at the end of the test.

should be removed by careful dissection of the tube.[23]

All dead and apparently dead individuals recovered by sieving should be examined closely under a dissecting microscope or handheld magnifying glass. Each of these individuals should be prodded gently to confirm no sign of life (such as a parapodial twitch), afterwhich it is to be counted as dead.

All live animals recovered from the overlying water or sediment in a single test chamber are counted and placed together in a numbered weighing pan or similarly small holding receptable, and rinsed for less than five seconds in deionized or distilled water to remove saltwater and any sediment adhering to the animal. After rinsing, the group of surviving worms should be transferred (with minimal water adhered to them) to a clean, aluminum weighing pan that has been previously numbered, weighed, and held in a desiccator.[24] Any remaining water evident in the weighing pan should then be removed using a disposable glass pipette, or by "wicking" the water with a fine artist's paint brush (while drying the paint brush using a paper towel).

Separate weighing pans, each containing the group of surviving animals recovered from each test chamber (replicate), are placed in an oven, and dried for 24 h at 60°C (EC, 1997a, b). Upon removal from the oven, the weighing pans are moved immediately to a desiccator. Following cooling, each pan should be removed from the desiccator, and weighed immediately[25] to the nearest 10 µg on a balance that measures accurately to this limit. Mean dry weight per individual worm which survived the test is calculated for each group (see Section 4.8).

During the series of dry-weight determinations for the groups of worms from a test, the first pan weighed should be replaced in the desiccator and weighed again at the end of all weighings, as a check on gain of water by the pans in the desiccator to be weighed subsequently. The change should not be >5%; if it is, redrying of all weighing pans for ≥2 h and reweighing might be carried out. A few weighing pans should be tared, dried, and weighed without animals, and results should conform to the laboratory's quality control standards (USEPA, 1994a; EC, 1997a, b).

4.8 Test Endpoints and Calculations

The biological endpoints for this 14-day *solid-phase sediment* toxicity test are survival and dry weight. Reduced survival and/or lesser weight at test end is assessed by comparison with replicate reference and/or control groups (see Sections 5.6 and 6.5). The most sensitive of the two effects is taken as the definitive indication of toxicity.

At the end of the 14-day exposure, the number of worms alive and number dead are recorded for each replicate including the control groups. Missing individuals are assumed to have died and disintegrated during the test, and are included in the tally of dead individuals for a replicate. The total dry weight of the group of survivors in a replicate is then measured.

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[23] It is very important to check all tubes (including broken ones) for the presence of worms, by gentle prodding. Otherwise, live worms might stay in their tubes and be counted as missing and presumed dead. It is also important to be gentle when sieving sediment and removing animals from their tubes, as they can fragment easily. The use of a fine artist's paint brush for prodding the tubes to remove the animals is recommended.

[24] It might be advisable to oven-dry the weighing pans for at least 48 h to achieve a constant weight, since wax deposits associated with the weighing pans could otherwise provide weighing errors (G.T. Ankley, personal communication, USEPA, Duluth, MN, 1994).

[25] The dried animals can take up water vapour readily, so weighing should be rapid and the time standardized among weighing pans. At the same time, care must be taken because rapid movement and static charge could cause dried specimens to be lost from the weighing pan.

The following two statistical endpoints must be calculated for each treatment:

- the mean (± SD) *percentage* of worms that survived the 14-day exposure; and

- the mean (± SD) dry weight per surviving worm, calculated from the total weight of the group of survivors.

The test is invalid if the average percent survival for the replicate groups of worms held in the negative control sediment for 14 days is <90% at the end of the test (ASTM, 1994; USACE, 1995).

Various statistical procedures can be used to assess the results of the test. The options, rationale for choice, and methods of calculation are discussed in depth in reports by the United States Environmental Protection Agency (USEPA, 1988, 1994a) and Environment Canada (EC, 2001c). The choice of statistical treatment depends on the test and study design and, in particular, whether tests used *replicate samples* of sediment or multiple concentrations of test substances or materials. Sections 5 and 6 provide guidance on statistical endpoints and calculations.

4.9 Tests with a Reference Toxicant

The routine use of a reference toxicant is necessary to assess, under standardized test conditions, the relative sensitivity of juvenile *P. cornuta* within one or more cultures from which test organisms are selected for use in one or more definitive sediment toxicity tests. Tests with a reference toxicant also serve to demonstrate the precision and reliability of data produced by the laboratory personnel for that chemical, under standardized test conditions. *Water-only test*s with a reference toxicant are most commonly used in conjunction with *survival-and-growth* tests which measure sediment toxicity to polychaete worms (USEPA, 1990b; ASTM, 1994; USACE, 1995) (see Appendix F). Procedures for *spiking* sediment with reference chemical(s) and for conducting *spiked sediment* reference toxicity tests, are available or being developed (Burton, 1991; Suedel *et al.*, 1993; EC, 1995) and should see wider use in the future.

In keeping with USEPA (1990b), ASTM (1994), USACE (1995), and Environment Canada (1997a, b), a *static*, 96-h *water-only* reference toxicity test using cadmium chloride is recommended here for routine use in conjunction with all sediment toxicity tests conducted using *P. cornuta*. This reference toxicity test may be supplemented or replaced with one or more *spiked-sediment* tests with reference toxicant(s) after suitable procedures are standardized. Environment Canada's guidance document on using negative control sediment spiked with a reference toxicant (EC, 1995) should be consulted if *spiked-sediment* reference toxicity tests are performed in conjunction with definitive sediment toxicity tests using *P. cornuta*.

Table 3 provides a checklist of conditions and procedures recommended for conducting static, 96-h *water-only* reference toxicity tests using *P. cornuta*. The recommended test procedure uses cultured worms that are 3 to 4 weeks' post-release to start the test. There are ten individuals per test chamber, at least five test concentrations plus a control (i.e., using control/dilution water only), and one or more replicates per treatment. Recommended test chambers are 250- or 300-mL high form glass beakers or jars with an inner diameter of ~7 cm, and the recommended test volume is 200 mL solution/chamber. Solutions in test chambers are not aerated during the test and are normally covered to minimize contamination and losses due to evaporation (EC, 1997a, b). Animals should not be fed during the test.

Temperature and lighting conditions for this test procedure are the same as those described for definitive sediment toxicity tests (see Section 4.2 and Tables 2 and 3). Daily observations are made for numbers of dead or moribund worms in each test chamber. Temperature, dissolved oxygen,

Table 3 Checklist of Recommended Conditions and Procedures for Conducting *Water-Only* Reference Toxicity Tests Using *Polydora cornuta*

Test type	–	static 96-h *water-only* toxicity test
Reference toxicant	–	cadmium chloride
Frequency of test	–	once per month, or in conjunction with definitive test(s) with sediment samples
Test solutions	–	control and at least five test concentrations
Solution replacement	–	none
Control/dilution water	–	culture water or other clean seawater; reconstituted seawater if a high degree of standardization is required; salinity, normally 28 ± 2‰; dissolved oxygen, 90 to 100% saturation when used in test
Test organisms	–	cultured juvenile *P. cornuta*, 3 to 4 weeks post-release at start of test; mean dry weight of individual worms must be within the range of 0.06 to 0.5 mg at start of test; 10 worms/test chamber
Substrate for test organisms	–	none
Test chamber	–	250-mL or 300-mL high form glass beaker or glass jar, ~7 cm ID, covered with lid or watchglass
Volume of test solution	–	200 mL
Number of replicates	–	one or more per concentration
Temperature	–	daily average, 23 ± 1°C; instantaneous, 23 ± 3°C
Lighting	–	overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark
Aeration	–	none
Feeding	–	none
Observations	–	daily, each chamber, for number of dead or moribund organisms
Measurements of water quality	–	start and end of test, each treatment, for DO, temperature, pH, and salinity
Endpoints	–	mean percent survival, each treatment; 96-h LC50
Test validity	–	results for reference toxicity test considered invalid if mean 96-h survival in control water <90%

pH, and salinity are measured for each treatment at the start and end of the test. The test endpoints are the mean percent survival in each treatment, and the 96-h LC50. Results for a reference toxicity test must be declared invalid if the mean survival in control water is <90% at the end of the test (Table 3).

Appropriate criteria for selecting suitable reference toxicant(s) might include the following (EC, 1990, 1995):

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- can be interspersed evenly throughout clean substrate;
- good dose/response curve for test organism;
- stable in aqueous solution;
- minimal hazard posed to user;
- concentration easily analyzed with precision;
- known influence of water quality (e.g., pH, salinity) on toxicity of chemical to test organism; and
- known influence of physicochemical characteristics of sediment (e.g., particle size, organic carbon content) on toxicity of chemical to test organism.

Reagent-grade cadmium chloride has been used repeatedly as a reference toxicant in conjunction with chronic sediment toxicity tests using *P. cornuta* or another species of marine or estuarine polychaete worm (USEPA, 1990b; ASTM, 1994; Arenicola Marine, 1994; Pocklington *et al.*, 1995; USACE, 1995); and is currently used routinely as a reference toxicant for sediment assays with marine or estuarine amphipods (EC, 1992a). *Water-only* reference toxicity tests using cadmium chloride must either be performed monthly with the laboratory's established cultures of *P. cornuta*, or a reference toxicity test must be conducted in conjunction with the definitive sediment toxicity test(s). The performance of any cultures recently established in the laboratory using new breeding stock should also be evaluated using this reference toxicant and the procedure given here, before these cultures are used to provide test organisms (see Section 2.3.1).

Each test with a reference toxicant should be performed using control/dilution water adjusted to a standard salinity, since this variable could otherwise influence the toxicity of cadmium from test to test. For reference toxicity tests using *P. cornuta*, a test salinity of 28 ± 2‰ is recommended.

Pertinent reports by Environment Canada provide guidance on the selection, performance, and use of *water-only* (EC, 1990) or *spiked-sediment* (EC, 1995) reference toxicity tests. Laboratory personnel unfamiliar with such tests are advised to consult these reports before preparing for or conducting them.

It is the laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with a reference toxicant before conducting definitive *survival-and-growth* sediment assays with *P. cornuta*. To meet this responsibility, the laboratory personnel should initially determine intralaboratory precision, expressed as coefficient of variation (CV), by performing five or more tests with the reference toxicant using different groups of *P. cornuta* taken from separate *known-age* cultures (Section 2.3.10) reared in the laboratory (USEPA, 1994a; EC, 1997a, b). For these preliminary tests, the same chemical concentrations, type/source of test water, and test procedure (as per Table 3) should be used. Performance of the routine (e.g., monthly) reference toxicity tests with cadmium chloride should continue to follow this same procedure. A series of test concentrations should be chosen[26], based on preliminary tests, to provide partial mortalities in two or more concentrations and

[26] See Appendix I for guidance in selecting an appropriate series of test concentrations. Each successive concentration chosen should be at least 50% of the previous concentration.

enable calculation of a 96-h LC50 with acceptably narrow confidence limits (see Section 6.5). Section 1.4 should be consulted for information on historic 96-h LC50s for cadmium chloride, derived in *water-only* reference toxicity tests with *P. cornuta* performed according to the procedures and conditions described herein.

Stock solutions of reagent-grade cadmium chloride should be made up on the day of use. They should be prepared using deionized water. A concentration of 1000 mg Cd/L is recommended. The concentration of cadmium in each stock solution (mg Cd/L) should be confirmed by analysis. Chemical analysis should be according to standard and recognized procedures (e.g., APHA *et al.*, 1998).

Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis if the LC50 was found to be outside warning limits. If stored before analysis, aliquots of test solutions and stock solution must be acidified (APHA *et al.*, 1998) and held in the dark at 4 ± 2°C. Any stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same test solutions at the end of the test, after completing biological observations. Calculations of LC50 should be based on the geometric mean measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

Once sufficient data are available (EC, 1990, 1995, 2001c), all comparable (i.e., same salinity and same source or type of control/dilution water) LC50s derived from these toxicity tests with cadmium chloride must be plotted successively on a *warning chart*, and examined to determine whether the results are within ± 2 SD of values obtained in previous comparable tests using the same reference toxicant and test procedure. A separate warning chart must be prepared and updated for each dissimilar procedure (e.g., differing salinity or differing source or type of control/dilution water). The warning chart should plot the logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with established limits of the chart; the LC50 is acceptable if it falls within the *warning limit*s.

The logarithm of concentration (including LC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each LC50 was estimated based on logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and ±2 SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the LC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable.

The mean of the available values of log(LC50), together with the upper and lower warning limits (±2 SD), should be recalculated with each successive LC50 for the reference toxicant until the statistics stabilize (EC, 1990, 1995, 2001c). If a particular LC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying LC50 would not necessarily indicate abnormal sensitivity of the culture or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all culturing and test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, to obtain new breeding stock, and/or to establish new *known-age* cultures, before undertaking further sediment toxicity tests.

Results that remained within the warning limits might not necessarily indicate that a laboratory was generating consistent results. Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in test results. A coefficient of variation of no more than 30%, and preferably 20% or less, is suggested as a reasonable limit by Environment Canada (1990). For this biological test method, the coefficient of variation for mean historic data derived for *water-only* reference toxicity tests performed using cadmium chloride should not exceed 30%.

Specific Procedures for Testing Field-Collected Sediment or Similar Particulate Material

This section gives particular instructions for preparing and testing samples of field-collected sediment or similar particulate material. These instructions are in addition to the procedures listed in Section 4.

Detailed guidance for the collection, handling, transport, storage, and analyses of field-collected sediment is given in Environment Canada (1994) and ASTM (1995a) reports specific to these subjects. Environment Canada (1994) should be consulted and followed (in addition to the guidance provided here), when collecting samples of field-collected sediment and preparing them for toxicity tests with cultured *P. cornuta*.

5.1 Sample Collection

Environment Canada (1994) provides a useful summary of field-sampling design and appropriate techniques for sample collection. Field surveys of sediment toxicity using biological tests with polychaete worms and/or other suitable, sediment associated test organisms are frequently part of more comprehensive surveys. Such surveys could include a *battery of toxicity tests* to evaluate the toxicity of sediment, pore water, or elutriate, together with tests for bioaccumulation of contaminants, chemical analyses, biological surveys of epifaunal and/or infaunal organisms, and perhaps the compilation of geological and hydrographic data. Statistical correlation can be improved and costs reduced if the samples are taken concurrently for these tests, analyses, and data acquisitions.

Samples of sediment collected for assessment of an adverse effect on survival and growth of cultured *P. cornuta* might be routinely taken (e.g., quarterly, semiannually, or annually) from a number of contaminated or potentially contaminated sites for *monitoring* and *compliance* purposes, or might be collected on one or more occasions during field surveys of sites for spatial (i.e., horizontal or vertical) or temporal definition of sediment quality. One or more sites should be sampled for *reference* (presumably clean) *sediment* during each field collection.[27]

The number of stations to be sampled at a study site and the number of *replicate samples* per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical constraints (e.g., time and cost) and statistical considerations. Environment Canada (1994) should be consulted for guidance with respect to the sampling design, including the recommended minimum number of field replicates.

For certain monitoring and regulatory purposes, multiple replicates (i.e., separate samples from different grabs or cores taken at the same site) should be taken at each *sampling station*,

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[27] A reference sediment is that collected near the site(s) of concern. Ideally, it possesses geochemical characteristics similar to those of the test sediment but without anthropogenic contaminants. It is not unusual for nearby reference sites to have some degree of contamination due to anthropogenic chemicals. In some instances, reference sediment might be toxic due to naturally occurring physical, chemical, or biological properties (Burton, 1991).

One or more samples of *reference sediment* should be included in each series of toxicity tests with *test sediment*(s). This sediment might or might not prove toxic due to the presence of naturally occurring chemicals such as hydrogen sulphide or ammonia, or the unanticipated presence of contaminants from human influence at harmful-effect concentrations. The use of such (toxic) sediment as *reference sediment* in future toxicity tests should be avoided, unless the experimental design is cognizant of this, and the investigator(s) wish to compare test results for this material with those for one or more samples of test sediment.

including one or more reference stations (EC, 1992a, 1994, 1997a, b; ASTM, 1994; USEPA, 1994a). Each of these field replicates should be tested for its toxicity to *P. cornuta*, using five or more test chambers per replicate sample. The use of *power analysis* statistics (see Section 5.6) with endpoint data obtained in previous *survival-and-growth* tests, performed with previous samples from the same or similar sites, will assist in determining if more than five laboratory replicates need to be tested with each field replicate. Also, some of the statistical tests have requirements for a minimum number of replicates. For certain other purposes (e.g., preliminary or extensive surveys of the spatial distribution of toxicity), the survey design might include only one sample from each station, in which case the sample would normally be homogenized and split between five or more test chambers. The latter approach precludes any determination of mean toxicity at a given sampling location (station), but allows a statistical comparison of the toxicity of each sample with the *reference sediment*(s) and/or *negative control sediment*, and also if desired, a comparison among the test samples (stations), using appropriate statistical tests.

Sites for collecting *reference sediment* should be sought where the geochemical properties of the sediment are similar to sediment characteristics encountered at the test sites. Matching of organic carbon content might not be warranted in cases where pollution (e.g., from pulp mills or sewage) is responsible for the high organic content of test sediments. Preliminary surveys to assess the toxicity and geochemical properties of sediment within the region(s) of concern and at neighbouring sites are useful for selecting appropriate sites at which to collect reference sediment.

Samples of municipal or industrial sludge (e.g., sewage sludge, dewatered mine tailings, or sludge from an industrial clarifier or settling pond) might be collected for assessment of adverse effects on the survival and growth of *P. cornuta*, and for geochemical and contaminant analyses. Other particulate wastes (e.g., drilling mud residue) might also be taken for toxicity and chemical evaluation.

Procedures used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives and the nature of the sediment or other particulate material being collected. The types of sediment collection devices and their advantages and disadvantages have been summarized by Environment Canada (1994), and further details are provided elsewhere (de Groot and Zschuppe, 1981; Baudo *et al.*, 1990; Burton, 1992; Sly and Christie, 1992; ASTM, 1995a).

A benthic grab or core rather than a dredge should be used for sampling sediment, to minimize disruption of the sample. Sediment to be evaluated for toxicity and chemistry should be collected from one or more depths that represent the layer(s) of concern (e.g., a surficial 2-cm layer, or a deeper layer if there are concerns about historical deposition of contaminants, or depending on the depth of any material to be dredged).

Care must be taken to minimize loss of fines during sample collection. If the sample is obtained using a grab sampler, hand corers should be used to collect a sample from the surficial 2 cm, or desired layer, of the test sediment retrieved using this sampling device. This can be achieved if the grab can be opened from the top to expose the surface of the undisturbed sediment. The sample should be transferred to a clean sample container.

Before commencing a sampling program, the required volume of sediment per sample should be calculated (EC, 1994). This calculation should take into account the quantity of sediment required to prepare laboratory replicates for sediment toxicity tests, as well as that required for particle size characterization, percent organic matter, percent moisture, and specific chemical analyses. A volume of at least 5 to 7 L of sediment per sample is normally required (EC,

1994), although this will depend on the study objectives/design and the nature of the chemical analyses to be performed. To obtain the required sample volume, it is frequently necessary to combine subsamples retrieved using the sampling device. Guidance provided in Environment Canada (1994) for compositing subsamples in the field should be followed.

The same collection procedure should be used at all field sites sampled. Environment (1994) should be consulted for further guidance on appropriate devices and procedures for sample collection.

5.2 Sample Labelling, Transport, Storage, and Analyses

Containers for transport and storage of samples of field-collected sediment or similar particulate material must be made of nontoxic material. The choice of container for transporting and storing samples depends on both sample volume and the potential end uses of the sample. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. The containers must either be new or thoroughly cleaned, and each should be rinsed with test water or other clean water (e.g., distilled or deionized water) before use.

Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed, and labelled or coded. Labelling and accompanying records made at this time must include at least a code or description which identifies sample type (e.g., grab, core, composite), source, precise location (i.e., water body, latitude, longitude, depth), replicate number, and date of collection; and should include the name and signature of sampler(s). Persons collecting samples of sediment should also keep records describing details of:

- the nature, appearance, and volume of each sample;
- the sampling procedure and apparatus;
- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each sampling station;
- the sampling schedule;
- the types and numbers of containers used for transporting samples;
- any field measurements (e.g., temperature, salinity, pH, dissolved oxygen) of the overlying water or sediment at the collection site; and
- procedures and conditions for cooling and transporting the samples.

Upon collection, warm (>7°C) samples should be cooled to between 1 and 7°C with regular ice or frozen gel packs, and kept cool (4 ± 3°C) in darkness throughout the period of transport. As necessary, gel packs, regular ice, or other means of refrigeration should be used to assure that the temperature of the sample(s) remains within 1 to 7°C during transit.

The date of receipt of the sample(s) at the laboratory must be recorded. Sample temperature upon receipt at the laboratory should also be measured and recorded. Samples to be stored for future use must be held in airtight containers and in darkness at 4 ± 2°C (EC, 1994, 1997a, b). Any air "headspace" in the storage container should be purged with nitrogen gas, before capping tightly (EC, 1994). Samples must not freeze or partially freeze during transport or storage, and must not be allowed to dry (EC, 1992a, 1994, 1997a, b). It is recommended that samples of sediment or similar particulate material be tested as soon as possible after collection. The sediment toxicity test should begin within two weeks of sampling, and

preferably within one week; the test must start no later than six weeks after sample collection.[28]

Ideally, sediment characteristics that are unstable (e.g., pH, hydrogen sulphide, oxidation-reduction potential) or changed by conditions of transit and storage (e.g., temperature) should be measured in the field to help characterize the sample. Field measurements of porewater salinity might, at certain sites, be advisable to assist in selecting sampling locations or in determining if porewater salinity is within the tolerance limits for this species of spionid worm (see Section 1.4) or other test organisms under consideration for exposure to the samples in laboratory sediment toxicity tests. In the laboratory, each sample of field-collected sediment should be thoroughly mixed (Section 5.3), and representative subsamples taken for physicochemical characterization. Each sample (including all samples of *negative control sediment* and *reference sediment*) must be characterized by analyzing subsamples for at least the following:

- for whole sediment — particle size distribution (percentage of coarse-grained sand, medium-grained sand, fine-grained sand, silt, and clay), percent water content, and total organic carbon content;
- for pore water — salinity, pH, and ammonia (total and un-ionized concentrations; see Section 4.6).

Other analyses could include (ASTM, 1994; USEPA, 1994a; APHA *et al.*, 1998): total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, oxidation-reduction potential, acid volatile sulphides, hydrogen sulphide, metals, synthetic organic compounds, oil and grease, organosilicones, petroleum hydrocarbons, and porewater analyses for various physicochemical characteristics. Unless indicated otherwise, identical chemical, physical, and toxicological analyses should be performed with subsamples representative of each replicate sample of field-collected sediment (including *reference sediment*) taken for a particular survey of sediment quality, together with one or more subsamples of *negative control sediment*.

5.3 Preparing Sample for Testing

With the exception of field-collected samples of negative control sediment (see Section 3.5), samples of field-collected sediment or similar particulate waste material must not be prepared for testing by sieving with water, as this would remove contaminants present in the pore water or loosely sorbed to particulate material (EC, 1994). Large debris or large indigenous macro-organisms should normally be removed using forceps or a gloved hand. If a sample contains a large quantity of debris (e.g., mollusc shells, wood chips, glass, plastic, gravel) or large macro-organisms, these may be removed by pressing the sediment through a coarse sieve (e.g., mesh size of ~5 mm). Water must not be used during any sieving of test sediments to remove undesirable material.

Unless research or special study objectives dictate otherwise, each sample of field-collected test material should be homogenized in the laboratory

[28] The toxicity and geochemistry of contaminated sediments from Hamilton Harbour was reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al*., 1990). A study by Othoudt *et al.* (1991) found that the toxicity of samples of freshwater sediment did not differ significantly when stored at 4°C for periods of 7 to 112 days. Burton (1991) and USEPA (1994a, b) report studies by various researchers showing in some instances that the toxicity of sediment held at 4°C was unchanged after several months' storage, and in other cases that changes were noted within days to weeks. A recommendation for testing within two weeks conforms with the advice in other sediment toxicity tests by Environment Canada (EC, 1992a, 1997a, b). A maximum permissible storage time of six weeks has been recommended by Environment Canada (EC, 1994) for sediments intended for toxicity tests, in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

before use (EC, 1994; USEPA, 1994a).[29] Mixing can affect the concentration and bioavailability of contaminants in the sediment, and sample homogenization might not be desirable for all purposes.

To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample may be stirred using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture and colour are homogeneous (EC, 1992a). Alternatively, a mechanical method (EC, 1994; USEPA, 1994a) may be used to homogenize the sample. For each sample included in a test, mixing conditions including duration and temperature must be as similar as possible. If there is concern about the effectiveness of sample mixing, subsamples of the sediment should be taken after mixing, and analyzed separately to determine homogeneity. Any moisture separating from a sample during its transport and storage must be remixed into it.

Immediately following sample mixing, subsamples of test material required for the toxicity test and for physicochemical analyses must be removed and placed in labelled test chambers (Section 4.1), and in the labelled containers required for storage of subsamples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using *P. cornuta* or other test organisms should also be transferred to labelled containers at this time. All subsamples to be stored should be held in sealed containers with no air space, and must be stored in darkness at 4 ± 2°C (Section 5.2) until used or analyzed. Each subsample must be thoroughly remixed to ensure homogeneity just before analysis or use in the toxicity test.

No attempt should be made to adjust the salinity of the interstitial water of test sediments from the field, using sieving or re-suspension in test water or any other means. Such manipulations might change the toxicological properties of the sediment and are unwarranted (ASTM, 1994). Rather, the test should be conducted using overlying water of the appropriate salinity (see Sections 3.4, 4.4, and 5.4).

5.4 Test Water and Salinity

For tests with field-collected sediment or similar particulate material, the seawater introduced to test chambers (i.e., overlying water) may be from the same source as that used for culturing the test organisms (see Sections 2.3.4 and 3.4). Alternatively, this water may be from a separate supply of natural or reconstituted seawater. For certain applications, the experimental design might require or endorse the use of brackish or full-strength seawater taken from nearby the reference site where test sediments were collected. Use of uncontaminated site water, or uncontaminated water adjusted to the salinity of site water, is frequently a good choice due to the modifying influence of waters with differing salinities on the toxicity of metals or organic contaminants in sediment. Sections 3.4 and 4.4 provide guidance and instructions regarding the preparation and analysis of seawater to be used as overlying water in tests with field-collected sediment or similar particulate material.

5.5 Test Observations and Measurements

A qualitative description of each field-collected test material should be made at the time that the test is being set up. This might include observations of sample colour, texture, and homogeneity; and the presence of plants, animals, and tracks or burrows of animals (EC, 1992a). Any changes in the appearance of the test material

[29] One of the reasons for routinely homogenizing samples is to mix into the sediment, any pore water which rises to the surface during sample shipment and storage. Homogenization is also necessary to redistribute the sample constituents that have compacted and layered according to particle size during transport and storage.

and in the overlying water, observed during the test or upon its termination, should be noted and reported.

Measurements of the quality of the overlying water (e.g., pH, temperature, salinity, ammonia, dissolved oxygen content) in test chambers should be made during or at the beginning and end of the test, as described in Section 4.6. Depending on the test objectives and experimental design, additional test chambers might also be set up at the beginning of the test (Section 4.1), to monitor whole sediment and/or porewater chemistry (EC, 1992a; ASTM, 1994; USEPA, 1994a). These test chambers would be destructively sampled during and/or at the end of the test. Test organisms might or might not be added to these extra test chambers, depending on study objectives. Measurements of chemical concentrations in the sediment or pore water within these chambers may be made by siphoning most of the overlying water without disturbing the surface of the sediment, then removing aliquots of the sediment for the appropriate analyses (see Section 5.2). If pore water were to be analyzed, centrifugation without filtration would be the recommended sampling procedure (EC, 1994; USEPA, 1994a). Environment Canada (1994) should be consulted for guidance on the recommended procedure for extracting pore water, and its treatment and storage before analyses.

Depending on study objectives and the nature of the test sediments (e.g., estuarine or rich in organics), measurements of porewater salinity, pH, and ammonia concentrations might be made as the test progresses, using test chambers dedicated for this purpose (USEPA, 1990b, 1994b; ASTM, 1994). Other sediment characteristics (e.g., concentrations of metals, hydrogen sulphide, total volatile solids, Eh) might be monitored in the same test chambers (ASTM, 1994). If it were desired to monitor these variables, at least one test chamber should be set up for each treatment, and destructively sampled for this purpose.

5.6 Test Endpoints and Calculations

The common theme for interpreting tests with one or more samples of field-collected *test sediment*, is a comparison of the biological effects for that test material with the effects found in a *reference sediment*. The reference sample should be used for comparative purposes whenever possible or appropriate, because this provides a site-specific evaluation of toxicity (USEPA, 1994a). Sometimes the reference sediment might be unsuitable for comparison because of toxicity or physicochemical characteristics dissimilar to the test sediment(s). In such cases, it would be necessary to compare the biological effects for the test sediments with the those for the negative control sediment. Results for the negative control sediment will assist in distinguishing contaminant effects from noncontaminant effects caused by such things as particle size and organic carbon content. Regardless of whether the reference sediment or negative control sediment is used for the statistical comparisons, the results from negative control sediment must be used to judge the validity and acceptability of the test (Section 4.2).

Analysis of results will differ according to the purposes and particular designs of the test. This section covers the analytical procedures, starting with the simplest design and proceeding to the more complex designs. Standard statistical procedures are generally all that is needed for analysing the results. Investigators should consult Environment Canada (2001c) as well as USEPA (1994a, Section 14) and USEPA/USACE (1994, Appendix D) for guidance on the appropriate statistical endpoints and their calculation. As always, the advice of a statistician familiar with toxicology should be sought for design and analysis of tests.

Analysis of variance (ANOVA) and multiple-comparison tests are commonly used for statistical interpretation of the significance of findings from sediment toxicity tests. This is an hypothesis-

testing approach, and is subject to the appreciable weaknesses described in Section 6.5.3. Notably, any increased variability within the test will weaken its power to distinguish toxic effects, resulting in an endpoint at a higher concentration (i.e., less toxicity is concluded). Similarly, use of only a few replicates instead of many replicates will weaken the discrimination of a test and will lead to a conclusion of less apparent toxicity, other things being equal (see Section 5.6.2). There is no alternative to hypothesis-testing for analysis of most toxicity tests with sediment, because only one concentration of sample, usually full strength (100% sample), is used. There are superior alternatives for point estimates of toxicity if multiple concentrations of each sample of field-collected sediment are tested (see Section 6).

The parametric analyses with ANOVA and multiple-comparison tests assume that the data are normally distributed, and that the variance is homogeneous among the different groups. As the first step in analysis, these assumptions should be tested with *Shapiro-Wilk's Test* for normality and *Bartlett's Test for Homogeneity of Variance* (Eisenhart *et al.*, 1947; Sokal and Rohlf, 1969). If the data are satisfactory by these tests, analysis may proceed. If not, data could be transformed (e.g., as square roots, logarithms, or as arcsine square root for *quantal* data which are to be used in quantitative analysis; Mearns *et al.*, 1986). The tests for normality and homogeneity might then show conformance with normality and homogeneity, and in fact that is a likely outcome of transformation.

Parametric tests are actually quite robust in the face of moderate deviations from normality and equality of variance. Therefore, parametric analysis (e.g., ANOVA and multiple comparison) should proceed, even if moderate nonconformity continues after transformation. Excluding a data-set for minor irregularities might lose a satisfactory and sensitive analysis and forgo the detection of real effects of toxicity.[30] Analysis by nonparametric tests should also proceed in parallel, with the more sensitive (lower endpoint) of the two analyses providing the final estimates of toxicity (see further explanation and rationale in Environment Canada, 2001c).

Multi-concentration tests might be conducted with one or more samples of field-collected sediment, sludge, or similar particulate material. Measured amounts of the test sample could be mixed with measured quantities of natural or artificial *negative control sediment* (see Sections 3.5 and 6.2), or with *reference sediment* (see Section 3.7). Procedures for mixing different samples of sediment are not yet standardized or proven (see Section 6.2), and caution is advised due to possible nonlinear responses and changes in bioavailability or sorption characteristics (Nelson *et al.*, 1994). A minimum of five subsamples are recommended to provide replicates in each concentration, to determine sample homogeneity and test precision. Guidance in Section 6 (including that in Section 6.5 for calculating test endpoints) should be followed if a multi-concentration test using one or more samples of field-collected *test sediment* diluted with *negative control sediment*, *clean* reference sediment, or any other sediment is performed.

5.6.1 Variations in Design and Analysis

A very preliminary survey might have only one sample of *test sediment* (i.e., contaminated or potentially contaminated sediment) and one

[30] Tests for normality and homogeneity become less meaningful with the small number of samples from individual sampling stations typically collected in studies of environmental toxicology. Plotting and examining the general nature of the distribution of toxicity and its apparent deviations can be more revealing and is recommended (EC, 2001c). Equality in sample sizes and the magnitude of variation are probably more important factors for the outcome of parametric analysis, but they have received scant attention in toxicology. Robustness of ANOVA is shown by its ability to produce realistic probabilities if the distribution of data is reasonably symmetrical, and if treatment variances are within threefold of each other (Newman, 1995).

sample of *reference sediment*, without replication. Simple inspection of the results might provide guidance for designing more extensive studies. If there were a single test sample and a single reference sample, with equal replication for each, a standard *Student's t-test* would be suitable for analysis (Paine and McPherson, 1991a). The t-test is fairly robust. The full formula for the t-test should be used, because it contains provisions for irregular data. The formula handles unequal numbers of replicates in the test and reference samples, as well as unequal variances in the two groups (USEPA/USACE, 1994). If there were *laboratory replicates* only, the interpretation of findings would be different from a survey in which there were *field replicates*, as described in the following paragraphs for *analysis of variance*.

A preliminary study might conceivably be run with samples from many stations, but without either field replicates or laboratory (within-sample) replicates. The objective might be to identify a reduced number of sampling stations deserving of more detailed and further study. Opportunities for statistical analysis would be limited. The nonreplicated test data could be compared with the reference data using outlier detection methods (USEPA, 1994a; Newman, 1995; EC, 2001c). A sample would be considered toxic if its result was rejected as an extreme value when considered as part of the data for the reference sediment and/or the *negative control sediment*.

A more usual survey of sediments would involve the collection of samples from several places by the same procedure(s), and their comparison with a single *reference sediment* and/or *negative control sediment*. There are several pathways for analysis, depending on the type and quality of data, but often there would be an *analysis of variance (ANOVA)* followed by one of the multiple-comparison tests. In the ANOVA, the *reference sediment* would also be treated as a "location".

In these multi-location surveys, the type of replication would make a difference in how the results were interpreted. There might be one sample at each of a number of locations, with *laboratory replicates* obtained by subdividing each sample. In that case, the one-way ANOVA would distinguish whether there was an overall difference among locations, a difference that was greater than the baseline variability in the *within-laboratory* procedures for setting up and running the test. Sampling variability would not really be assessed in the statistical analysis, except that it would contribute to any difference found in locations. If *field replicates* were collected at each of the sampling locations, and no laboratory replicates were used, the same type of one-way ANOVA would evaluate overall difference in locations, over and above the *combined* variability of sampling the location and running the test. It would be much more powerful to have field replicates for all sampling locations, and also laboratory replicates of each field replicate. If that were done, the laboratory replicates would become the replicates in a nested one-way ANOVA, and would be the base of variability for comparing differences in the samples. The ANOVA could be used to see (a) if there was an overall difference in locations, and (b) whether there was an overall difference in replicates taken at the various locations. After an ANOVA, the analysis would proceed to one or more type of multiple-comparison test, as described in the following.

After the ANOVA for multi-location surveys, different statistical tests are available for application. If it were desired to compare each sampling location with the reference to see if the two were different, *Dunnett's test* should be used. This statistical test assumes normality and equal variance, and is based on an *experiment-wise* value of α (the probability of declaring a significant difference when none actually exists). If replication were unequal, investigators should seek the advice of a statistician, or adopt the complete interlocation comparison described in the following paragraph.

In a multi-location survey, an investigator might wish to know which sampling locations showed results that differed statistically from which others, as well as knowing which ones were different from the reference and/or control. Such a situation might involve a number of locations "downstream" of an effluent discharge or other point source of contamination, in which instance the investigator might want to know which locations had significantly higher toxicity than others and were particularly deserving of cleanup. *Tukey's test* is designed for such an analysis, and is commonly found in statistics packages. This test can deal with unequal sample sizes.[31]

If it were desired to compare each sampling location with the reference, but the data did not conform to requirements of normality and equal variance, the ANOVA and subsequent tests would be replaced by nonparametric tests. *Steel's Many-One Rank test* would be used if replication were equal, while unequal replication would require *Wilcoxon Rank Sum test with Bonferroni's adjustment*.

5.6.2 Power Analysis

An important factor to consider in the analysis of toxicity tests with sediment is the potential for declaring false "positives" (i.e., calling a *clean* site dirty) or false "negatives"(i.e., calling a dirty site *clean*). Scientists are usually cautious in choosing the level of significance (α) for tolerating false positive results (Type I error), and usually set it at P = 0.05 or 0.01. Recently, toxicologists have been urged to report both α and statistical power ($1 - \beta$), i.e., the probability of correctly rejecting the null hypothesis (H_0) and not making a Type II error. There are several factors that influence statistical power, including:

- variability of replicate samples representing the same treatment;
- α (i.e., the probability of making a Type I error);
- *effect size (ES)*, (i.e., the magnitude of the true effect for which you are testing); and
- *n* (i.e., the number of samples or replicates used in a test).

Power analysis can be used *a priori* to determine the magnitude of the Type II error and the probability of false positive results. It can also be used to ascertain the appropriate number of field and laboratory replicates for subsequent surveys involving this test, or to assist in the selection of future sampling sites. It is always prudent to include as many replicates in the test design as is economically and logistically warranted (see Section 5.1); power analysis will assist in this determination. A good explanation of the power of a test, and how to assess it, can be found in USEPA (1994a), with some coverage in EC (2001c).

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[31] An alternative approach is currently recommended for sediment testing in the United States (USEPA, 1994a; USEPA/USACE, 1994). For equal replicates, *Fisher's Least Significant Difference (LSD)* is said to be a superior approach. It is based on a smaller "pairwise error rate" for α in comparing any given location with another, but holds the overall value of α to the pre-selected value (usually 0.05). *LSD* is seldom included in software packages for toxicity, but it is described in some textbooks (e.g., Steel and Torrie, 1980), and is detailed in Appendix D of USEPA/USACE (1994). *Tukey's test* is instead recommended here, partly because *LSD* might declare significant differences too readily. *LSD* is also intended for only a few of all the possible comparisons in a set of data, and those comparisons would have to be specified in advance. In the current US recommendations for pairwise comparison, a situation with unequal replicates would call for *Bonferroni's adjustment of the t-test* to replace testing by *LSD* (USEPA, 1994a). That adjusted t-test enables all possible comparisons among locations.

Specific Procedures for Testing Chemical-Spiked Sediment

This section gives guidance and instructions for preparing and testing *negative control sediment*, *reference sediment*, or any other sediment spiked experimentally with chemical(s) or chemical product(s). These recommendations and instructions are in addition to the procedures listed in Section 4. More detailed and appropriate guidance for spiking sediment with chemical(s) and conducting toxicity tests with chemical/sediment mixtures is given in Environment Canada (1995). Further evaluation and standardization of procedures for spiking sediment (Section 6.2) might be required before sediment toxicity tests with spionid polychaete worms (i.e., *P. cornuta*) or other appropriate test organisms are applied to evaluate specific chemical/sediment mixtures for regulatory purposes.

The cause(s) of sediment toxicity and the interactive toxic effects of chemical(s) or chemical product(s) in association with otherwise *clean* sediment can be examined experimentally by spiking *negative control sediment* (Section 3.5) or *reference sediment* (Section 3.7) with these substances. The spiking might be done with one or more chemicals or chemical products. Toxicity tests using sediment spiked with a range of concentrations of test chemical(s) or test product(s) can estimate LC50s (see Section 6.5.1), and can determine threshold concentrations causing specific sublethal effects (see Sections 6.5.2 and 6.5.3).

Procedures are described herein for preparing test mixtures of chemical-spiked sediment (Section 6.2), making observations and measurements during and at the end of the toxicity test (Section 6.4), and estimating test endpoints for multi-concentration tests (Section 6.5). These procedures also apply to the mixing of multiple concentrations of field-collected *test sediment* (including particulate waste material such as sludge or dredged sediment intended for ocean disposal) in *negative control sediment* or *reference sediment*, and to performing multi-concentration tests and determining statistical endpoints for these mixtures (see Section 5, and especially 5.6). Multi-concentration tests with *positive control sediment* (see Section 3.6) or one or more reference toxicants spiked in *negative control sediment* (see Sections 3.5 and 4.9) are performed using the procedures and statistical guidance described in this section. Additionally, the influence of the physicochemical characteristics of natural or artificial *negative control sediment* (or other sediment) on chemical toxicity can be determined with spiked-sediment toxicity tests according to the procedures and statistical guidance described in this section.

6.1 Sample Properties, Labelling, and Storage

Information should be obtained on the properties of the chemical(s) or chemical product(s) to be spiked experimentally in *negative control sediment, reference sediment*, or other sediment. For individual chemicals, chemical products (e.g., pesticides or other commercial formulations), or chemical mixtures thereof, available information should be obtained on the concentration of major ingredients and impurities, water solubility, vapour pressure, chemical stability, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Where aqueous solubility is in doubt or problematic, acceptable procedures previously used for preparing aqueous solutions of the chemical(s) should be obtained and reported, and chemical solubility in test water should be determined experimentally. Other available information such as structural formulae, nature and percentage of significant impurities, presence and amounts of additives, and n-

octanol:water partition coefficient, should be obtained and recorded.

Chemical(s) to be tested should be at least reagent grade, unless a test on a formulated commercial product or technical grade chemical(s) is required (USEPA, 1994a). Chemical containers must be sealed and coded or labelled upon receipt. Required information (chemical name, supplier, date received, person responsible for testing, etc.) should be indicated on the label and/or recorded on a separate datasheet dedicated to the sample, as appropriate. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical.

6.2 Preparing Test Mixtures

Differing procedures have been used by researchers to spike (dose) *clean* negative control sediment or reference sediment with chemical(s) or chemical product(s) (Burton, 1991; USEPA, 1994a, b; ASTM, 1995a, b). Mixing technique and time, as well as the period of aging after mixing, can affect the toxicity of the mixture (USEPA, 1994a, b).

Investigators may choose to use either artificial sediment or natural (field-collected) sediment from an uncontaminated (*clean*) site, as the *negative control sediment* (see Section 3.5) to be spiked with chemical(s) or chemical product(s) and for the corresponding replicates of negative control sediment to be included in the test. Depending on the study design and objectives, a *clean* reference sediment or a contaminated or potentially contaminated sediment (e.g., from a particular site under investigation) might also be spiked with one or more chemicals or chemical products to determine the influence of such test substance(s) on sediment toxicity.

Experimental procedures (including those for substance addition and mixing, as well as equilibration time and conditions) used to prepare each *batch* of chemical-spiked sediment are new, varied, and not standardized. Accordingly, a standardized methodology for preparing chemical-spiked sediment cannot be recommended at this time. Rather, some of the approaches used previously or thought to be reasonable for preparing chemical-spiked sediment for toxicity tests with polychaete worms are given here.

Environment Canada (1994, 1995) reports provide more detailed instructions and recommendations for spiking and homogenizing sediment, and should be consulted for further guidance. Researchers intending to pursue toxicity tests using one or more laboratory-prepared mixtures should proceed cautiously, and should be well aware of potential problems due to non-homogeneity of the mixture(s) and the associated changes in bioavailability/sorption characteristics and nonlinear toxic responses that might result (Nelson *et al.*, 1994).

The salinity of the interstitial water in sediments spiked experimentally with contaminants in the laboratory may be adjusted to a desired value, before spiking. To accomplish this, the negative control (or other) sediment to be used for preparing the chemical/sediment mixture should be sieved using test water with the desired salinity (see Sections 3.4 and 3.5).

The procedure to be used for experimentally spiking sediment is contingent on the study objectives and the nature of the test substance to be mixed with *negative control sediment* or other sediment. In many instances, a chemical/sediment mixture is prepared by making up a *stock solution* of the chemical(s) or chemical product(s) and then mixing one or more measured volumes into artificial or natural *negative control sediment* (Swartz *et al.*, 1985, 1988; USEPA, 1994a, b; EC, 1995). Chemical concentrations in sediment are frequently calculated and expressed as μg/g or mg/kg dry weight (Swartz *et al.*, 1985, 1988), although concentrations based on wet weight might be more useful for relating results to sediment toxicity (Burton, 1991). Depending on the nature of the test substance or material and test objectives, concentrations might also be

normalized to sediment organic carbon content (e.g., for evaluating the toxicity of nonpolar organic compounds) or to acid volatile sulphides (e.g., for assessing metal toxicity) (Di Toro *et al.*, 1990, 1991; USEPA, 1994a, b).

The preferred solvent for preparing stock solutions is *test water* (see Sections 2.3.4 and 3.4); use of a solvent other than water should be avoided unless it is absolutely necessary. For test chemical(s) or chemical product(s) that do not dissolve readily in test water, a suitable water-miscible organic solvent of low toxicity may be used to help disperse the compound in water (EC, 1992a, 1997a, b; ASTM, 1994, 1995b; USEPA, 1994a, b). Triethylene glycol has been recommended because of its low toxicity to aquatic organisms, low volatility, and high ability to dissolve many organic chemicals (ASTM, 1994). Other solvents such as dimethylsulphoxide, methanol, ethanol, or acetone may also be used to prepare stock solutions of organic chemicals, although they might contribute to sample toxicity, alter sediment properties, or be lost from the test material due to their volatility. Surfactants should not be used (EC, 1992a, 1997a, b; ASTM, 1994).

If an organic solvent is used, the test must be conducted using a series of replicate test chambers containing only *negative control sediment* (i.e., 100% artificial or natural *clean* sediment containing no solvent and no test substance), as well as a series of replicate test chambers containing *solvent control sediment*. For this purpose, a *batch* of *solvent control sediment* must be prepared which contains the concentration of solubilizing agent that is present in the highest concentration of the test chemical(s) or chemical product(s) in sediment. Solvent from the same batch used to make the stock solution of test substance(s) must be used (EC, 1992a, 1997a, b; ASTM, 1994; USEPA, 1994a, b).

Solvents should be used sparingly as they might contribute to the toxicity of the prepared test sediment. The maximum concentration of solvent in the sediment should not affect the survival or growth of worms during the test. If this lack of effect is unknown or uncertain, a preliminary *solvent only* test, using various concentrations of solvent in *negative control sediment*, should be conducted to determine the threshold-effect concentration of the particular solvent being considered for use in the definitive test.

Measured volumes of a stock solution containing test chemical(s) or chemical product(s) should be mixed with negative control (or other) sediment to achieve a homogeneous distribution of the test substance(s) throughout the sediment. Mixing may be by hand (e.g., using a clean spatula or glass rod), or by using a mechanical stirring or mixing device (e.g., Ditsworth *et al.*, 1990). Alternatively, the test substance(s) can be coated on the walls of a flask and an aqueous slurry (i.e., a mixture of *negative control sediment* and *test water*) added. The flask contents are then mixed by agitation. Another alternative is to add a measured volume of the stock chemical solution directly to a slurry of negative control (or other) sediment in test water, agitate the mixture, and allow it to settle (EC, 1992a, 1997a, b). Other procedures for mixing might prove to be acceptable provided that the chemical is shown to be evenly distributed in the sediment. Mixing conditions, including solution:sediment ratio, mixing and holding time, and mixing and holding temperature, must be standardized for each treatment included in a test. Time for mixing a chemical-spiked sediment should be adequate to ensure homogeneous distribution of the chemical, and may be from minutes up to 24 h. During mixing, temperature should be kept low to minimize changes in the mixture's physicochemical characteristics and microbial activity. Analyses of subsamples of the mixture are advisable to determine the degree of mixing and homogeneity (Ditsworth *et al*., 1990; ASTM, 1994; USEPA, 1994a, b; EC, 1997a, b).

For some studies, it might be necessary to prepare only one concentration of a particular mixture of negative control (or other) sediment and

chemical(s) or chemical product(s), or a mixture of only one concentration of contaminated sediment or particulate waste in negative control or other sediment. For instance, a single-concentration test might be conducted to determine whether a specific concentration of chemical or chemical product in *clean* sediment is toxic to the test organisms. Such an application could be used for research or regulatory purposes.

A multi-concentration test, using a range of concentrations of chemical added to *negative control sediment* (or other sediment) under standardized conditions, should be used to determine the desired endpoints (i.e., *LC50*, *ICp*, *NOEC*, *LOEC*; see Section 6.5) for the chemical/sediment mixtures. A multi-concentration test using *negative control sediment* spiked with a specific particulate waste might also be appropriate. At least five test concentrations plus a control must be prepared for each multi-concentration test; and the preparation and use of six to ten concentrations (plus one or more negative control sediments) is recommended for certain tests to improve the likelihood of attaining each endpoint sought. When selecting the test concentrations, an appropriate geometric dilution series may be used in which each successive concentration of chemical(s) or chemical product(s) in sediment is at least 50% of the previous one (e.g., 10, 5, 2.5, 1.25, 0.63 mg/kg).[32] Test concentrations may also be selected from other appropriate logarithmic dilution series (see Appendix I).

Tests intended to evaluate the toxicity of mixtures of test substance(s) in negative control sediment (or other sediment) for federal registration or other regulatory purposes must be set up using a minimum of five replicates for each test concentration and each negative control sediment to be included in the assay. Since the objective for a multi-concentration test is to determine both LC50 (mortality data) and ICp (dry weight data), a test using six to ten concentrations plus the control(s) is recommended.

To select a suitable range of concentrations, a preliminary or range-finding test covering a broader range of test concentrations might prove worthwhile. The number of replicates per treatment could be reduced or eliminated altogether for range-finding tests and, depending on the expected variance among test chambers within a treatment, might also be reduced for nonregulatory screening bioassays or research studies.

It is recommended that mixtures of spiked sediment be aged for four weeks before starting a test, in keeping with a common practice (USEPA, 1994a, b; ASTM, 1995b; EC, 1997a, b). Although many studies with chemical-spiked sediment have been started within a few hours or days of preparing the mixtures, such short and variable time periods might not be long enough for equilibration of the chemicals mixed in negative control (or other) sediment. A consistent four-week period of aging a mixture before initiating a toxicity test would provide some standardization for intra- and interlaboratory comparisons of results for tests with chemical-spiked sediment. Once prepared, each mixture should be placed in a suitable, sealed (with no air space) container, and stored in the dark at 4 ± 2°C (Section 5.2) for four weeks before use (EC, 1997a, b).

Based on the objectives of the test, it might be desirable to determine the effect of substrate characteristics (e.g., particle size or organic content) on the toxicity of chemical/sediment mixtures. For instance, the influence of sediment particle size on chemical toxicity could be measured by conducting concurrent multi-concentration tests with a series of mixtures comprised of the test chemical(s) or chemical product(s) mixed in differing fractions (i.e., segregated particle sizes) or types of natural or

[32] Concentrations in sediment are normally calculated and expressed as µg/g or mg/kg, based on either dry weight or wet weight. In some instances, concentrations in pore water might also be measured and expressed as µg/L or mg/L.

artificial *negative control sediment* (Section 3.5). Similarly, the degree to which the organic content of sediment can modify chemical toxicity could be examined by performing concurrent multi-concentration tests using different chemical/sediment mixtures prepared with a series of organically enriched negative control sediments. Each fraction or formulation of natural or artificial *negative control sediment* used to prepare such mixtures should be included as a control in the test.

Tests could be required to measure the effect on survival and weight of *P. cornuta* at test end, caused by one or more concentrations of specific chemical(s) or chemical product(s) introduced to the test chamber as a solution overlying the sediment. Procedures for preparing test concentrations could vary depending on the objectives of the study. One approach would be to carefully add the test solution(s) to replicate chambers containing a layer of *negative control sediment* or other sediment (e.g., field-collected *test sediment* from a particular site), with no disturbance or subsequent mixing of the sediment and test solution(s). A second approach would require the test solution(s) introduced to test chambers to be agitated for a predetermined period in the presence of the sediment before the test organisms are introduced. Chemical/sediment interactions might differ appreciably depending on the approach taken, and could result in a markedly different test result. Unless specified or otherwise required, equilibrated *test water* (Sections 3.4 and 6.3) should be used to prepare each test solution. Replicate controls, including solvent controls if a solvent is used, must be prepared and treated identically. Instructions provided earlier in this section regarding the use of solvents other than water should be followed in preparing solvent controls.

6.3 Test and Control/Dilution Water

Normally, *clean test water* should be used for preparing stock or test solutions of chemicals and as test water in spionid *survival-and-growth* tests with mixtures of chemical-spiked sediment (see Section 3.4). The source of this water may be reconstituted seawater or natural seawater, and might or might not be identical to the water used for culturing the test organisms (see Section 2.3.4). For comparative tests requiring a high degree of standardization, reconstituted or natural seawater adjusted to one or more fixed, narrow salinity ranges (e.g., 15 ± 1‰ for estuarine water; 28 ± 1‰ for "full-strength" seawater) is recommended. For further standardization (e.g., in instances where the toxicity of one or more chemical/sediment mixture is to be measured and compared at a number of test facilities), the use of a standard reconstituted water, prepared using the same formulation (and, in some instances, batch) of reagent-grade salts or commercial sea salts (USEPA, 1994c, 1995; EC, 2001b), is recommended.

6.4 Test Observations and Measurements

A qualitative description of each mixture of chemical-spiked sediment and of the overlying test water should be made when the test is being established. This might include observations of the colour, texture, and visual homogeneity of each mixture of chemical-spiked sediment, and observations of the colour and opacity of the overlying water. Any change in appearance of the test mixture or overlying water noted during the test, or upon its termination, should be recorded. Measurements of the quality of each mixture of chemical-spiked sediment being tested (including the *negative control sediment*), and of the overlying water, should be made and recorded as described in Sections 4.6, 5.2, and 5.5.

If analytical capabilities permit, it is recommended that the stock solution(s), overlying water, sediment, pore water, and test solutions (if studied) be analyzed together with one or more aliquots of each spiked-sediment mixture, to determine the chemical concentrations, and to assess whether the sediment has been spiked satisfactorily. These should be preserved, stored,

and analyzed according to suitable, validated procedures.

Unless there is good reason to believe that the chemical measurements are not accurate, toxicity results for any tests in which concentrations are measured for each spiked-sediment mixture included in the test should be calculated and expressed in terms of those average measured concentrations determined for both the whole sediment (µg/kg or mg/kg, dry weight) and the pore water (µg/L or mg/L). In cases where concentrations of chemical(s) or chemical product(s) added to the overlying water are being tested, results should again be expressed as the average measured concentrations determined for the sediment and the pore water, although average chemical concentrations measured for the test solutions overlying sediment should also be calculated and reported (EC, 1992a, 1997a, b).

As a minimum, sample aliquots should be taken from the high, medium, and low test concentrations at the beginning and end of the test, in which instance endpoint values calculated (Section 6.5) would be based on nominal ones. Any such measurements of concentrations of the test chemical(s) or chemical product(s) should be compared, reported, and discussed in terms of their degree of difference from nominal strengths.

6.5 Test Endpoints and Calculations

Multi-concentration tests with mixtures of spiked sediment are characterized by the 14-day LC50 and an endpoint representing the weight data (e.g., ICp). Appropriate statistics and programs for calculating these endpoints are summarized in this section. Section 5.6 provides guidance for calculating and comparing endpoints for single-concentration tests performed with mixtures of chemical-spiked sediment. For further information on the appropriate parametric or nonparametric statistics to apply to the endpoint data, the investigator should consult the Environment Canada report on statistics for the determination of toxicity endpoints (EC, 2001c) as well as USEPA (1994a; Section 14), USEPA (1994b; Section 12), USEPA (1994c), USEPA (1995), or USEPA/USACE (1994).

For any test that includes *solvent control sediment* (see Section 6.2), the performance of test organisms in that sediment must be compared statistically with that in *negative control sediment*. If any of the endpoints for these two control sediments differ according to Student's *t*-test, only the *solvent control sediment* may be used for comparison and calculation of results. If the results for the two controls are the same, the data from both controls should be combined before use in calculating results or assessing test validity.

6.5.1 Median Lethal Concentration (LC50)

When a multi-concentration test with spiked sediment mixtures is conducted (Section 6.2), the *quantal* mortality data must be used to calculate (data permitting) the 14-day *median lethal concentration (LC50)*, together with its 95% confidence limits. To estimate an LC50, mortality data at 14 days are combined for all replicates at each concentration. If mortality is not ≥50% in at least one concentration, the LC50 cannot be estimated. If there are no mortalities at a specific concentration, that information is used as an effect of 0% mortality. However, if successive concentrations yield a series of 0% mortalities, only the highest concentration of the series should be used in estimating the LC50 (i.e., the zero-effect that is "closest to the middle" of the distribution of data). Similarly, if there were a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, i.e., the one at the lowest concentration. Use of only one 0% and one 100% effect applies to any form of statistical analysis and to hand plotting on a graph.

Various computer programs may be used to calculate the LC50. Guidance provided in EC (2001c) should be followed when choosing and applying the appropriate statistical program for this calculation. Stephan (1977) developed a program to estimate LC50s using probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. Other

satisfactory computer and manual methods may be used (e.g., USEPA, 1985b, 1988; Hubert, 1987; APHA *et al.*, 1998). Programs using the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended unless "zero trim" is specified, since divergent results might be obtained by operators who are unfamiliar with the implications of trimming ends of the dose-response data (EC, 2001c). A statistical method for calculating the LC50 using logistics is now emerging as a preferred approach (EC, 2001c).

Any computer-derived LC50 should be checked by examining a plot, on logarithmic-probability scales, of percent mortalities at Day 14 for the various test concentrations (APHA *et al.*, 1998; EC, 2001c). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved. A hand-plotted graph is preferred for this check. A computer-generated plot could be used if it were based on logarithmic-probability scales. If there had been an error in entering the data, however, a computer-generated plot would contain the same error as the mathematical analysis, and so the investigator should carefully check for correct placement of points.

A manual plot of mortality/concentration data to derive an estimated LC50 is illustrated in Figure 2. In this hypothetical example, there were 25 worms (five replicates of 5 organisms each, per concentration) tested at each of five concentrations. This figure was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg chemical/kg sediment causing mortalities of 0, 20, 40, 90, and 100% of test organisms exposed to the respective concentrations for 14 days. The concentration expected to be lethal to 50% of the worms can be read by following across from 50% (broken line) to the intersection with the best-fit line, then down to the horizontal axis for an estimated LC50 (5.6 mg/kg). A similar plot could be made of mortality/concentration data using the average measured concentration (in µg/L or mg/L) determined for the porewater analyses (see Section 6.4).

In fitting a line such as that in Figure 2, more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper (*log-probit*, as in Figure 2) can be purchased in good technical bookstores, ordered through them, or photocopied (see blank graph in EC, 2001c).

For the regular set of data in Figure 2, computer programs gave very similar estimates to the graphic one. The LC50s (and 95% confidence limits) were:

Stephan (1977) method:
- probit 5.58 (4.24 and 7.37)
- moving average 5.58 (4.24 and 7.33)
- binomial 6.22 (between 1.8 and 10)

Probit analysis of Hubert (1987): 5.56 (4.28 and 7.21)

TOXSTAT 3.4: 5.58 (4.38 and 7.12)

SAS (1988) probit analysis: 5.58 (4.26 and 7.40)

6.5.2 Inhibiting Concentration for a Specified Percent Effect (ICp)

For the data on mean dry weight, the *ICp* (*inhibiting concentration for a specified percent effect*) is the recommended statistical endpoint. The ICp is a *quantitative* estimate of the concentration causing a fixed percent reduction in mean dry weight of worms (e.g., the IC25 and/or IC20, which represent 25% and 20% reduction). The desired value of p is selected by the investigator, and 25% or 20% is currently favoured. Any ICp that is calculated and reported must include the 95% confidence limits.

The mean weight of worms is calculated as the total dry weight of the organisms that survived in a given test chamber, divided by the number of organisms that survived to the end of the test (Section 4.7). If there are no survivors in a

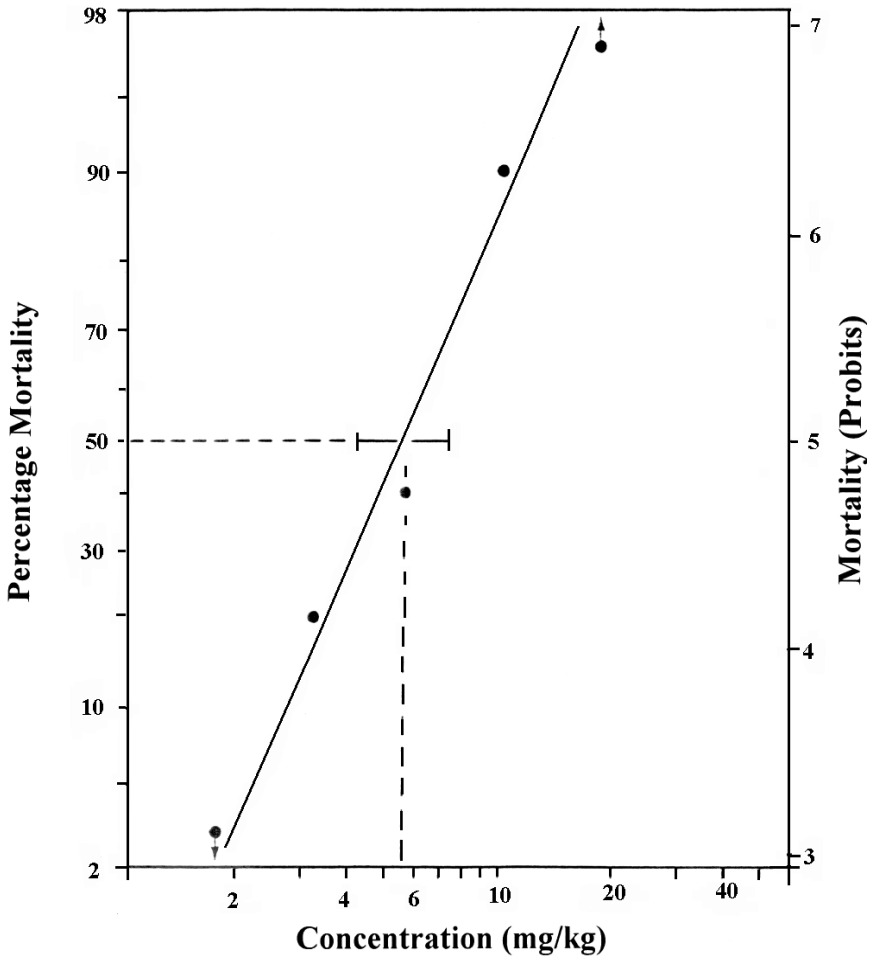

Figure 2 Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-Probability Paper

replicate (chamber), that replicate is excluded from the analysis. If there is complete mortality in all replicates at a given concentration, that concentration is excluded.

At present, the only easily available method of estimating the ICp and its 95% confidence limits is the "bootstrap" method on computer (Norberg-King, 1993); a program called *ICPIN* (USEPA, 1994c; 1995). ICPIN is not proprietary, is available from USEPA, and is included in most computer software for environmental toxicology, including TOXSTAT. The original instructions for ICPIN from USEPA are clearly written and make the program easy to use (Norberg-King, 1993).[33] An earlier version was called BOOTSTRP.

Analysis by ICPIN does not require equal numbers of replicates in different concentrations. The ICp is estimated by smoothing of the data as necessary, then using the two data-points adjacent to the selected ICp (USEPA, 1994c, Appendix L; USEPA, 1995, Appendix L). The ICp cannot be

[33] The instructions in Norberg-King (1993) are sometimes misleading on the identity of "replicates". The term is used in such a way that it would apply to weights of individual organisms within the same chamber. This slip of wording does not affect the functioning of the program.

calculated unless there are test concentrations both lower and higher than the ICp; both those concentrations should have an effect reasonably close to the selected value of p, preferably within 20%. At present, the computer program does not use a logarithmic scale of concentration, and so Canadian users of the program must enter the concentrations as logarithms. Some commercial computer packages have the logarithmic transformation as a general option, but investigators should make sure that it is actually retained when proceeding to ICPIN. ICPIN estimates confidence limits by a special "bootstrap" technique because usual methods would not be valid. Bootstrapping performs many resamplings from the original measurements. The investigator must specify the number of resamplings, which can range from 80 to 1000. At least 400 is recommended here, and 1000 would be beneficial.[34]

Besides determining and reporting a computer-derived ICp, a graph of percent reduction of dry weight against the logarithm of concentration should be plotted, to check the mathematical estimation and to provide a visual assessment of the nature of the data (EC, 2001c).

6.5.3 Hypothesis Testing (NOEC and LOEC)

An optional approach for presenting the results of the test is determining the *no-observed-effect concentration (NOEC), lowest-observed-effect concentration (LOEC),* and *threshold-observed-effect concentration (TOEC)*. For these spiked-sediment tests, NOECs and LOECs are calculated from the mean dry weights of surviving worms in each replicate (i.e., each test chamber) of the control and the various concentrations. Calculations use the same sublethal data as in estimating the ICp. If there is complete mortality in a replicate or a concentration, it is excluded from the analysis.[35] Statistical procedures are explained with some guidance in USEPA (1994a, c; 1995), USEPA/USACE (1994), Newman (1995), and EC (2001c), and in commercial software packages such as TOXSTAT (WEST, Inc. and Gulley, 1996). The methods start with a check of normality and homogeneity of variance by the *Shapiro-Wilks* and *Bartlett's tests*. If both tests are satisfied by the data in their original or transformed state (Section 5.6), analysis should proceed using parametric methods.

For parametric testing, an *analysis of variance (ANOVA)* is carried out, followed by *Williams' test*, a multiple-comparison test that determines which concentrations are significantly different from the control (Williams, 1971, 1972). Williams' test takes into account the order of each group of measurements according to their magnitude of concentration (EC, 2001c); this is a desirable feature to increase sensitivity, and a very appropriate attribute for most toxicity tests (Masters *et al.*, 1991).[36] Williams' test estimates the *Minimum Significant Difference (MSD)*. This is the magnitude of the difference in mean weights that would have to exist between the control and a test concentration to conclude that there was a

[34] ICPIN has some deficiencies. Its interpolation method is an inefficient use of data, sensitive to peculiarities of the two concentrations used. The program fails to adopt logarithm of concentration, which would introduce a slight bias towards a higher value of ICp. A modification of the bootstrap method has now remedied a problem of overly narrow confidence limits. Linear regression or general-purpose regression would be better methods of estimating the ICp and its 95% confidence limits (EC, 2001c), but a standard "packaged" method of regression has not been developed for environmental toxicology. Investigators should watch for any development of such a suitable new program.

[35] It is conceivable that significant mortality might occur at lower concentrations than those affecting mean weight. In this case, mortality is a more sensitive endpoint than growth, and the 14-day LC50 should be calculated.

[36] Another standard multiple-comparison test, *Dunnett's test*, is given more prominence in TOXSTAT and most methods from the United States. It is not a particularly powerful way of discriminating effects since it ignores the magnitudes of the concentrations when it calculates the MSD (Masters *et al.*, 1991).

significant effect at that concentration. Any test which reports NOEC/LOEC must also report the MSD. If the MSD of average weights is >25% of the mean weight of the controls, the validity and usefulness of the findings are questionable.

If there are unequal numbers of replicates because of accidental loss or other causes, Williams' test is replaced by the *Dunn-Sidak modification of the t-test*, or by *Bonferroni's adjustment of the t-test*.

If tests for conformity and homogeneity cannot be satisfied by transformation, the parametric analysis should proceed except in cases of severe departure from normality which clearly would not fit such an analysis. The parametric tests are relatively robust in the face of moderate nonconformance (see Section 5.6). Nonparametric analysis should also proceed. The more sensitive (lower concentrations) of the two analyses is to be used as the final estimate of NOEC and LOEC. In this situation, detailed reporting must include the following items:

- results of the *Shapiro-Wilks* and *Bartlett's tests*;
- a hand-plotted graph of mean weights by chamber, using logarithmic concentration;
- findings of parametric analysis, including MSD; and
- findings of non-parametric analysis.

Nonparametric analysis requires four replicates.[37] *Shirley's test* would be the method of choice instead of analysis of variance. It parallels *Williams' test* in taking into consideration the ranking of concentration. Unfortunately, *Shirley's test* is not available in most statistical packages, nor is it described in most textbooks. *Steel's many-one rank test* is offered in most United States statistical packages, and could be used in this situation; it does not consider the order of concentrations. If there were unequal replication, the *Wilcoxon rank sum test* should be used. These tests are strong tools for data that are not normally distributed, but they would be less powerful than parametric tests if used on normally distributed data.

The *geometric mean* of the NOEC and LOEC, the *TOEC*, is often calculated for the convenience of having one number. The TOEC should be reported, recognizing that it is an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. Its value is governed by whatever concentrations were selected for the test. No confidence limits can be estimated for the NOEC, LOEC, or TOEC.

[37] The requirement for four replicates might prevent an estimation of NOEC/LOEC. A test might have been designed with fewer replicates, primarily for calculating the ICp. If results were found to deviate from normality or homogeneity, the investigator would not be able to complete the analysis by nonparametric methods.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the *must* requirements delineated in Sections 2 to 6 of this biological test method, and, if so, provide details of deviation. 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 use intended.

Section 7.1 provides a list of items that must be included in each test-specific report. A list of 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, is found in Section 7.2. Specific monitoring programs, related test protocols, or regulations might require selected test-specific items listed in Section 7.2 (e.g., details about the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as *data to be held on file*.

Procedures and conditions common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or *compliance* purposes) and consistent with specifications herein, may be referred to by citation or by attachment of a general report outlining standard laboratory practice.

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

- a record of the chain-of-continuity for field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on 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 toxicity tests;
- detailed records of the source of the test organisms, their taxonomic confirmation, and all pertinent information regarding their culturing and health; and
- information on the calibration of equipment and instruments.

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

7.1 Minimum Requirements for a Test-Specific Report

The following sections list items that must be included in each test-specific report.

7.1.1 Test Substance or Material

- brief description of sample type (e.g., dredged material, reference or contaminated field-collected sediment, negative control sediment) or coding, as provided to the laboratory personnel;

- information on labelling or coding of each sample; and
- date of sample collection; date and time sample(s) received at test facility.

7.1.2 Test Organisms

- species and source of brood stock and test organisms;
- range of age, at start of test;
- dry weight (mean ± SD), at start of test; and
- any unusual appearance, behaviour, or treatment of the organisms, before their use in the test.

7.1.3 Test Facilities

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

7.1.4 Test Water

- type, source, and salinity of test water; and
- measured characteristics of test water, before and/or at time of commencement of the toxicity test.

7.1.5 Test Method

- citation of biological test method used (i.e., as per this document);
- design and description if specialized procedure (e.g., preparation of mixtures of spiked sediment; preparation and use of solvent and, if so, solvent control) or modification of standard test method described herein;
- brief description of frequency and type of all measurements and all observations made during test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

7.1.6 Test Conditions and Procedures

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number of discrete samples per treatment; number of replicate test chambers for each treatment; number and description of treatments in each test including the control(s); test concentrations (if applicable);
- depth and volume of sediment and overlying water in each test chamber;
- number of organisms per test chamber and treatment;
- feeding regime and ration;
- dates when test was started and ended;
- for each sample — all measurements of sediment particle size, percent water content, and total organic carbon; and of porewater salinity, pH, and ammonia; and
- for at least one test chamber representing each treatment — all measurements of temperature, dissolved oxygen, salinity, ammonia, and pH in overlying water.

7.1.7 Test Results

- for each treatment — mean ± SD for percentage of worms that survived the 14-day exposure; mean ± SD for dry weight of individual surviving worms at test end; results of any statistical comparisons;
- coefficient of variation (CV) for mean percent survival and mean individual dry weight of replicate control groups at test end;

- any LC50 (including the associated 95% confidence limits and, if calculated, the slope) determined;

- any ICp (together with its 95% confidence limts) determined for the data on growth (i.e., dry weight at test end); details regarding any transformation of data that was required, and indication of *quantitative* statistic used;

- for a multi-concentration test with chemical-spiked sediment, indication as to whether results are based on nominal or measured concentrations of chemical(s) or chemical product(s); all values for measured concentrations;

- results for any 96-h LC50 (including its 95% confidence limits) performed with the reference toxicant in conjunction with the definitive sediment toxicity test, using the same batch of test organisms, reported as mg Cd/L; together with the geometric mean value (± 2 SD) for the same reference toxicant, test species, and salinity, as derived at the test facility in previous tests using the procedures and conditions herein; and

- anything unusual about the test, any problems encountered, any remedial measures taken.

7.2 Additional Reporting Requirements

The following sections list 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.

7.2.1 Test Substance or Material

- identification of person(s) who collected and/or provided the sample;

- records of sample chain-of-continuity and log-entry sheets; and

- conditions (e.g., temperature, in darkness, in sealed container) of sample upon receipt and during storage).

7.2.2 Test Organisms

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;

- history and age of brood stock, for any culture used to provide test organisms;

- description of culture conditions and procedures for *mixed-age* and *known-age* cultures, including: facilities and apparatus, lighting, water source and quality, water *pretreatment*, water exchange rate and method, water temperature and salinity, type and quantity of substrate);

- procedures used to count, handle, sort, transfer, and sieve animals; and those to determine their mortality, condition, appearance, and behaviour; and

- source and composition of food, procedures used to prepare and store food, feeding method(s), and feeding frequency and ration.

7.2.3 Test Facilities and Apparatus

- description of laboratory's previous experience with this biological test method for measuring sediment toxicity using *P. cornuta*;

- description of systems for providing lighting and compressed air, and for regulating temperature within test facility;

- description of test chambers and covers;

- description of apparatus and procedure used to deliver and renew overlying water in test chambers; and

- description of procedures used to clean or rinse test apparatus.

7.2.4 Negative Control Sediment and Test Water

- procedures for pretreatment of *negative control sediment* (e.g., sieving, settling of sieved fines, formulation and aging if artificial) and *test water* (e.g., filtration, sterilization, reconstitution and aging if reconstituted, salinity adjustment, temperature adjustment, aeration rate and duration);

- type and quantity of any chemical(s) added to test water; and

- storage conditions and duration before use.

7.2.5 Test Method

- procedures used for mixing or otherwise manipulating test sediments before use; time interval between preparation and testing;

- procedure used in preparing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;

- methods used (with citations) for chemical analyses of test material (sediment and pore water); including details concerning aliquot sampling, preparation, and storage before analysis; and

- use and description of preliminary or range-finding test.

7.2.6 Test Conditions and Procedures

- measurements of light intensity adjacent to surface of overlying water in test chambers;

- statement concerning the aeration of overlying water in test chambers before and during the test, including aeration rate and manner;

- records of any disruption of air flow to test chambers during test, and of related DO measurements;

- description of procedure and rate for renewal of overlying water;

- appearance of each sample (or mixture thereof) and of the overlying water in test chambers; changes in appearance noted during test;

- any other chemical measurements (e.g., contaminant concentration, acid volatile sulphides, biochemical oxygen demand, chemical oxygen demand, total inorganic carbon, cation exchange capacity, redox potential, porewater hydrogen sulphide, porewater ammonia) made before and during the test on test material (including control and reference sediment) and contents of test chambers; including analyses of whole sediment, pore water, and overlying water;

- any other observations or analyses made on the test material (including samples of *negative control sediment* or *reference sediment*); e.g., faunal tracks, qualitative and/or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses; and

- chemical analyses of concentration of chemical in stock solutions of reference toxicant and, if measured, in test concentrations.

7.2.7 Test Results

- results for any range-finding test(s) conducted;

- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant;

- 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.

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Appendix A

Biological Test Methods and Supporting Guidance Documents Published by Environment Canada's Method Development & Applications Section[1]

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996
Acute Lethality Test Using Threespine Stickleback (*Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21	February 1992	November 1997
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22	February 1992	November 1997
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24	November 1992	—
Growth Inhibition Test Using the Freshwater Alga *Selenastrum capricornutum*	EPS 1/RM/25	November 1992	November 1997
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27	December 1992	November 1997
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)	EPS 1/RM/28 1st Edition	December 1992	January 1995
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—

[1] These documents are available for purchase from Environmental Protection Publications, Environmental Protection Service, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. For further information or comments, contact the Manager, Method Development & Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)	EPS 1/RM/32	December 1997	—
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37	March 1999	—
B. Reference Methods[2]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	—
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	—
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	—
Guidance Document on Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	EPS 1/RM/30	September 1995	—
Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology	EPS 1/RM/34	December 1999	—

[2] For this series of documents, a *reference method* is defined as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of test instructions and conditions which are described precisely in a written document. Unlike other generic (multi-purpose or "universal") biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations.

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Appendix D

Procedural Variations for Culturing Marine or Estuarine Polychaete Worms in Preparation for *Survival-and-Growth* Tests of Sediment Toxicity, as Described in Published Methodology Documents

Source documents are listed here chronologically, by originating agency rather than by author(s).

USEPA 1990b represents the standard protocol for conducting a 20-day test for survival and growth of juvenile *Neanthes* in sediment; co-authored by D.M. Johns, T.C. Ginn, and D.J. Reish and prepared by PTI Environmental Services for the United States Environmental Protection Agency (USEPA), Region 10. This test method was published by USEPA in June 1990 (see "USEPA 1990b" in list of references).

ASTM 1994 represents the standard guide for conducting sediment toxicity tests with marine and estuarine polychaetes; written by D.J. Reish for the American Society for Testing and Materials (ASTM), under the jurisdiction of ASTM Subcommittee E47.03 on sediment toxicology, and published in July 1994 (see "ASTM 1994" in list of references). Procedures described in this test-method document specific to culturing *Neanthes arenaceodentata* in preparation for conducting 20- to 28-day survival-and-growth tests of sediment toxicity are summarized herein.

USACE 1995 represents an overview of the standard protocol used by researchers with the United States Army Corps of Engineers (Waterways Experiment Station, Vicksburg, MS) for conducting a 28-day test for survival and growth of juvenile *Nereis (Neanthes) arenaceodentata* in sediment. This test method was co-authored by T.M. Dillon, D.W. Moore, and T.S. Bridges, (see "USACE 1995" in list of references).

1 Species and Source of Brood Stock for Culture

Document	Species	Initial Source
USEPA 1990b	*Neanthes* sp.	from a culture maintained at another laboratory
ASTM 1994	*N. arenaceodentata*	natural (intertidal sand-mud flats or subtidal), or from a culture maintained at another laboratory[a]
USACE 1995	*N. arenaceodentata*	from a culture maintained at California State University, Long Beach, California

[a] All individuals used in a test should be from the same source.

2 Culture Chambers and Loading

Document	Chamber Type and Size	Water Volume (L)	Maximum Number of Animals per Culture Chamber
USEPA 1990b	glass aquarium	NI[a]	NI
ASTM 1994	aquarium, 37- to 57-L	NI	75–100
USACE 1995	38-L glass aquarium	30	NI

[a] Not indicated.

3 Water Source, Salinity, and Method of Replacement During Culturing

Document	Water Source	Salinity (‰)	Method of Replacement
USEPA 1990b	NI[a]	NI[b]	IR[c] (monthly), or FT[d]
ASTM 1994	natural or recon.[e]	NI[a]	IR[c] (monthly[f])
USACE 1995	reconstituted	30	NI[a]

[a] Not indicated.
[b] One to two days before test initiation, worms should be held in water at the salinity of that to be used in the toxicity test.
[c] Intermittent renewal.
[d] Flow-through.
[e] Reconstituted seawater, prepared using a commercially available sea salt or specified amounts of reagent-grade chemicals.
[f] Nearly all of the water in the aquarium should be changed once per month.

4 Temperature, Aeration, and Lighting During Culturing

Document	Water Temperature (°C)	Aeration Conditions	Lighting Conditions
USEPA 1990b	NI[a,b]	NI[a,c]	NI[a]
ASTM 1994	18–24	aerate[d]	NI[a]
USACE 1995	20	aerate	12-h light:12-h dark

[a] Not indicated.

[b] One to two days before test initiation, worms should be held in water maintained at a temperature of 20 ± 1°C (the culturing temperature preceding this holding period is not specified).

[c]
[d] One to two days before test initiation, worms should be held in an aquarium containing clean seawater that is aerated gently. Two airstones, at opposite sides of the aquarium, provide an adequate DO supply and water circulation.

5 Substrate for Worms During Culturing

Document	Description of Substrate Used
USEPA 1990b	worms are maintained without sediment; enough powdered alga (sieved to <0.3 mm) sufficient to cover the bottom of the aquarium is provided to enable tube construction[a]
ASTM 1994	sediment is not required to culture this species
USACE 1995	2–3 cm layer of fine-grained, uncontaminated marine sediment

[a] Use of a layer of powdered alga increases survival in cultures.

6 Feeding During Culturing

Document	Description of Food Used	Quantity and Feeding Frequency
USEPA 1990b	TetraMarin™ and powdered alga (*Enteromorpha* or *Ulva* sp.)	NI[a,b]
ASTM 1994	various (TetraMarin™, powdered alfalfa flour, powdered *Ulva* or *Enteromorpha* sp., or commercial rabbit food)	provide ~1.5 to 2.5 g of dried food once per week, depending on the size and number of worms[c]
USACE 1995	TetraMarin™ and alfalfa (finely ground to ≤0.50 mm and added as seawater slurry)	100 mg TetraMarin™ and 50 mg alfalfa added twice per week

[a] Not indicated.

[b] During the holding period (1 to 2 days) preceding a test, juvenile worms are provided ~8 mg (dry weight) TetraMarin™ per individual, every other day.

[c] Soak dry food in seawater before feeding.

Procedural Variations for *Survival-and-Growth* Tests of Sediment Toxicity Using Marine or Estuarine Polychaete Worms, as Described in Published Methodology Documents

Source documents are listed here chronologically, by originating agency rather than by author(s).

USEPA 1990b represents the standard protocol for conducting a 20-day test for survival and growth of juvenile *Neanthes* in sediment; co-authored by D.M. Johns, T.C. Ginn, and D.J. Reish and prepared by PTI Environmental Services for the United States Environmental Protection Agency (USEPA), Region 10. This test method was published by USEPA in June 1990 (see "USEPA 1990b" in list of references).

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USACE 1995 represents an overview of the standard protocol used by researchers with the United States Army Corps of Engineers (Waterways Experiment Station, Vicksburg, MS) for conducting a 28-day test for survival and growth of juvenile *Nereis (Neanthes) arenaceodentata* in sediment. This test method was co-authored by T.M. Dillon, D.W. Moore, and T.S. Bridges(see "USACE 1995" in list of references).

1 Test Type and Duration; Life Stage and Age of Test Organisms at Start

Document	Species	Test Type	Test Duration (days)	Life Stage and Age at Start
USEPA 1990b	*Neanthes* sp.	IR[a]	20	juveniles, 2–3 weeks post-release[b]
ASTM 1994	*Neanthes* sp.[c]	IR	20–28	juveniles, 2–3 weeks post-release
USACE 1995	*Neanthes arenaceodentata*	IR	28	juveniles, 2–3 weeks old

[a] Intermittent renewal.
[b] Worms should be 0.5–1.0 mg dry weight to ensure that they are in a rapid growth phase.
[c] *N. arenaceodentata* is used for 20- to 28-day survival-and-growth tests of sediment toxicity. This species or *N. virens* may also be used for a 10-day toxicity test which measures survival only.

2 Test Chambers and Materials

Document	Test Chamber	Cover	Amount of Sediment	Amount of Seawater
USEPA 1990b	1-L glass jar, with 10-cm ID	lid with hole	2-cm layer	NI[a] (fill chamber)
ASTM 1994	1-L glass beaker, with 10-cm ID	11.4-cm glass[b]	2-cm layer	NI (fill to 750-mL mark)
USACE 1995	1-L glass beaker	glass[b]	2- to 3-cm	NI (fill to layer 800-mL mark)

[a] Not indicated.
[b] Watchglass.

3 Number of Organisms per Chamber, Number of Samples per Station, and Number of Replicates per Station or Treatment

Document	Number Animals per Chamber	Number Samples per Station	Number Replicates per Station or Treatment
USEPA 1990b	5	1	5[a]
ASTM 1994	5	5[b]	5[c]
USACE 1995	5	NI[d]	5

[a] Typically, five replicate test chambers should be included for each sediment tested, including all control and reference sediments.

[b] Replicates should be taken at each station in the survey. Each survey should include five replicate samples from an area free of contamination (i.e., reference sediment).

[c] The number of replicates necessary per station is a function of the need for resolution. Subsamples from the same grab or composite should not be considered true replicates for statistical comparisons among stations. For spiked-sediment tests, five replicates should typically be included for each treatment (i.e., five replicates of each concentration tested, plus five replicates of control sediment).

[d] Not indicated.

4 Type and Treatment of Seawater Used as Overlying Water in Test

Document	Type and Treatment
USEPA 1990b	uncontaminated seawater; maintain at a salinity of 28 ± 2‰
ASTM 1994	natural or reconstituted; filter (≤5 µm) to remove suspended particles or organisms; if pathogens, treat with UV sterilization or filtration (≤0.45 µm); cover and store at 4 ± 3°C; use within 2 d if natural; if reconstituted, use high-quality water (not chlorinated or dechlorinated), age if necessary for 1 to 2 weeks, and aerate intensively before use
USACE 1995	30‰ seawater

5 Storage and Characterization of Sediment Used in Test

Document	Storage Condtions	Characteristics Measured[a]
USEPA 1990b	4°C in dark, for ≤14 d	NI[b]
ASTM 1994	4 ± 3°C in dark, for ≤2 weeks	at least S, pH, OC, W, SSC, HS, AMM; might include BOD, COD, Eh, TIC, M, SOC, OG, OS, PH
USACE 1995	4°C in dark, in sealed containers	grain size, porewater salinity, pH, ammonia

[a] S = porewater salinity; pH = hydrogen-ion concentration; OC = organic carbon; W = % water; SSC = % sand, silt, and clay; HS = hydrogen sulphide; AMM = total ammonia; BOD = biochemical oxygen demand; COD = chemical oxygen demand; Eh = oxidation reduction potential; TIC = total inorganic carbon; M = metals; SOC = synthetic organic compounds; OG = oil and grease; OS = organosilicones; PH = petroleum hydrocarbons

[b] Not indicated.

6 Manipulation of Sediment Before Use in Test

Document	Sediment Manipulation
USEPA 1990b	might be mixed with high-salinity water to raise porewater salinity, when testing sediment collected from low-salinity areas
ASTM 1994	sieve control sediment twice (e.g., 0.5 mm); do not wet-sieve field-collected sediment; remove any large organisms with forceps; thoroughly homogenize each sample; place aliquot (2-cm layer) in test chamber, smooth, gently add overlying seawater, cover chamber and aerate seawater overnight
USACE 1995	press-sieve (2 mm screen) without the addition of seawater; then homogenize thoroughly

7 Salinity, Temperature, and Lighting During Test

Document	Salinity (‰)	Temperature (°C)	Lighting
USEPA 1990b	28 ± 2[a]	20 ± 1	continuous, using ambient light of low to moderate intensity
ASTM 1994	depends on objective[b]	20 ± 1	polychaetes generally do not require a definite light regime
USACE 1995	30	20	12-h light:12-h dark

[a] Caution should be used if performing and interpreting the results of tests for sediments with an interstitial salinity <20‰.

[b] The optimum salinity for *Neanthes arenaceodentata* is 28–36‰. Use of this species for testing sediment from areas of low salinity is limited. If test sediments are collected from low-salinity areas, the salinity of the overlying water in the test chambers should be about the same as that of the interstitial water or the water above the sediment at the collection site.

8 Apparatus and Conditions for Aeration During Test

Document	Apparatus	Conditions
USEPA 1990b	glass Pasteur pipette	suspend pipette 3-4 cm below water surface; aerate continuously at 150 to 300 mL/minute
ASTM 1994	glass pipette with 1-mL capacity	suspend pipette ≥2 cm from sediment; aerate overnight before start of test, and throughout test; rate minimal, to maintain DO ≥90% without disturbance of sediment
USACE 1995	glass pipette	suspend pipette 2-3 cm above sediment; use trickle-flow aeration after any suspended sediment has settled

9 Renewal of Overlying Water During Test

Document	Frequency of Renewal	Procedure for Renewal
USEPA 1990b	every 3rd day	remove pipette, siphon out 1/3 of the volume of overlying water, replace it with fresh seawater adjusted to test temperature and salinity, replace pipette and adjust air flow
ASTM 1994	NI[a]	NI
USACE 1995	before start, and weekly	remove ~80% of overlying water and then refill to 800-mL mark

[a] Not indicated.

10 Feeding During Test

Document	Description of Food Used	Quantity per Chamber	Feeding Frequency
USEPA 1990b	TetraMarin™ fishfood flakes	40 mg	every 2nd day
ASTM 1994	NI[a]	NI	NI
USACE 1995	TetraMarin™ and alfalfa	5 mg TM[b] + 2.5 mg alf[c]	twice weekly[d]

[a] Not indicated.
[b] TetraMarin™.
[c] Alfalfa.
[d] Fed as a seawater slurry, after every renewal of overlying water and 3–4 days later.

11 Replacing and Observing Organisms During Test

Document	Replacing and Observing Organisms
USEPA 1990b	at 1 h after start, replace any unburrowed worms likely damaged or not healthy; observe daily for burrowing activity
ASTM 1994	at 1 h after start, replace any unburrowed worms likely damaged or not healthy
USACE 1995	at start, inspect each worm to ensure the inclusion of the correct number of undamaged worms to each test chamber

12 Monitoring Quality of Overlying Water During Test

Document	Variables Monitored[a]	Frequency
USEPA 1990b	DO, salinity, pH	just before replacement (i.e., every 3rd day), each chamber
ASTM 1994	temp	daily[b,c]
	salinity	daily
	DO, pH, amm	beginning and end[b,d]
USACE 1995	temp	daily
	DO, salinity, pH, amm	just before replacement and at end of test

[a] DO = dissolved oxygen; pH = hydrogen ion concentration; temp = temperature; amm = ammonia.
[b] Measured in at least one test chamber representing each treatment.
[c] Daily mean temperature should be within ±1°C of desired temperature; instantaneous temperature should be within ±3°C of desired temperature.
[d] If air flow to test chambers interrupted >1 h, measure DO.

13 Monitoring Quality of Sediment During Test

Document	Variables Monitored[a]	Frequency
USEPA 1990b	porewater salinity	beginning and end, each sediment
ASTM 1994	Eh and pH optional: M, TVS, etc.	beginning and end NI[b]
USACE 1995	NI	NI

[a] Eh = oxidation reduction potential; M = metals; TVS = total volatile solids.
[b] Not indicated.

14 Terminating Test and Biological Endpoints

Document	Terminating Test	Biological Endpoints
USEPA 1990b	sieve (0.5 mm) or sort sediment; rinse; remove worms in tubes; count survivors in each replicate; rinse; measure total dry weight for survivors in each replicate[a]	mean (± SD) % survival[b] mean (± SD) dry weight[c]
ASTM 1994	sieve (0.5 mm) or sort sediment; rinse; remove worms in tubes; count survivors in each replicate; rinse; measure total dry weight for survivors in each replicate[a]	mean (± SD) % survival[b] mean (± SD) dry weight[d] mean (± SD) growth rate[e]
USACE 1995	sieve (2.0, 1.0, 0.5 mm mesh) sediment; rinse; count survivors in each replicate; measure total dry weight for survivors in each replicate	% survival in each replicate; individual dry weight/ replicate; growth rate[e]

[a] Determine dry weight for each group of survivors to nearest 0.1 mg.
[b] Calculate for each treatment.
[c] Calculate for each treatment, both as total and individual biomass.
[d] Calculate for each treatment, as individual biomass.
[e] Calculate as mean individual growth rate (mg/day) for each treatment.

15 Use of Control/Reference Sediment and Requirement for Valid Test

Document	Control/Reference Sediment	Requirement for Valid Test
USEPA 1990b	control and reference sediments should be included as part of every test	NI[a]
ASTM 1994	every test requires one or more control or reference sediments[b]	mean control survival ≥90%[c]
USACE 1995	negative control or reference sediment	mean control survival ≥90%[d]

[a] Not indicated.

[b] At least five laboratory replicates of clean control sediment should be included in all tests. The design of field surveys should include an additional field control (i.e., reference sediment) involving five replicate samples from an area free of sediment contamination.

[c] For a test to be valid, survival in each replicate control chamber must also be ≥80%, and survival of animals during the 48-h period preceding the test must be ≥95%.

[d] For a test to be valid, survival in each replicate control chamber must also be ≥80%.

Procedural Variations for Reference Toxicity Tests Performed using Marine or Estuarine Polychaete Worms, in Conjunction with Published *Survival-and-Growth* Tests of Sediment Toxicity

Source documents are listed here chronologically, by originating agency rather than by author(s).

USEPA 1990b represents the standard protocol for conducting a 20-day test for survival and growth of juvenile *Neanthes* in sediment; co-authored by D.M. Johns, T.C. Ginn, and D.J. Reish and prepared by PTI Environmental Services for the United States Environmental Protection Agency (USEPA), Region 10. This test method was published by USEPA in June 1990 (see "USEPA 1990b" in list of references).

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USACE 1995 represents an overview of the standard protocol used by researchers with the United States Army Corps of Engineers (Waterways Experiment Station, Vicksburg, MS) for conducting a 28-day test for survival and growth of juvenile *Nereis (Neanthes) arenaceodentata* in sediment. This test method was co-authored by T.M. Dillon, D.W. Moore, and T.S. Bridges (see "USACE 1995" in list of references).

1 Reference Toxicant(s), Test Type, Duration, and Frequency of Use

Document	Reference Toxicant(s)	Test Type	Test Duration	Frequency of Use
USEPA 1990b	cadmium chloride	water only[a]	96 h	all tests[b]
ASTM 1994	metal or PAH[c]	water only	96 h	all tests[b]
USACE 1995	cadmium chloride	water only	96 h	all tests[b]

[a] Animals are exposed in clean, filtered seawater without sediment.
[b] Conduct in conjunction with all definitive growth-and-survival tests for sediment toxicity.
[c] A metal such as cadmium chloride or a polynuclear aromatic hydrocarbon such as fluoranthene might be used.

2 Life Stage of Test Animals, Test Chamber, Volume of Test Solution, Number of Animals per Chamber, and Number of Replicates per Treatment

Document	Life Stage of Animal	Test Chamber	Volume of Solution	Number of Animals per Chamber	Number of Replicates
USEPA 1990b	juvenile	NI[a]	NI[a]	NI[a]	NI[a]
ASTM 1994	NI[b]	NI[b]	NI[b]	NI[b]	NI[b]
USACE 1995	juvenile[c]	1-L bkr[d]	800 mL	5	5

[a] Not indicated. Use the same procedure and conditions as for toxicity tests with sediment.
[b] Not indicated.
[c] Animals for use in this test are taken from the same pool used to initiate the sediment-toxicity test.
[d] Glass beaker.

3 Source and Salinity of Water Used in Test, and Variables Monitored

Document	Water Source or Type	Salinity (‰)	Variables Monitored	Monitoring Frequency
USEPA 1990b	clean, filtered seawater	NI[a]	NI[a]	NI[a]
ASTM 1994	NI[b]	NI[b]	NI[b]	NI[b]
USACE 1995	NI[b]	30	cadmium	start and end of test

[a] Not indicated. Use the same procedure and conditions as for toxicity tests with sediment.
[b] Not indicated.

4 Temperature, Aeration, and Lighting During Test with Reference Toxicant

Document	Water Temperature (°C)	Aeration Conditions	Lighting Conditions
USEPA 1990b	NI[a]	NI[a]	NI[a]
ASTM 1994	NI[b]	NI[b]	NI[b]
USACE 1995	20	gentle; using glass pipette	12-h light:12-h dark

[a] Not indicated. Use the same procedure and conditions as for toxicity tests with sediment.
[b] Not indicated.

5 Feeding During Test with Reference Toxicant

Document	Description of Food Used	Quantity per Chamber	Feeding Frequency
USEPA 1990b	NI[a]	NI[a]	NI[a]
ASTM 1994	NI[b]	NI[b]	NI[b]
USACE 1995	do not feed	none	none

[a] Not indicated. Use the same procedure and conditions as for toxicity tests with sediment.
[b] Not indicated.

6 Endpoints and Requirement for Valid Test with Reference Toxicant

Document	Biological Endpoints	Statistical Endpoints	Requirement for Valid Test
USEPA 1990b	survival	LC50	NI[a]
ASTM 1994	NI[a]	NI[a]	NI[a]
USACE 1995	survival	LC50	NI[b]

[a] Not indicated.

[b] Not indicated. Data will be used to construct a Shewart Control Chart, and to identify "out of control" data and potentially invalid test results.

Systematics of *Polydora cornuta* (Bosc, 1802)

Taxonomy and Phyletic Relationship

Polydora cornuta belongs to the phylum Annelida, that includes those animals which are worm-like and have many similar segments (annulations). *P. cornuta* is related to the common earth worm (another annelid and a member of the class Oligochaeta, which has few bristles), but it belongs to the class of annelids characterized by having many bristles or setae - the Polychaeta. *P. cornuta* also belongs to the order Spionida and the family Spionidae. The family Spionidae contains approximately 32 genera and 320 species of marine or estuarine polychaete worms (Pettibone, 1982).

Members of the family Spionidae are generally sedentary, tube-building polychaete worms. The basic body parts of the family are labelled in Figure G.1a. The prostomium is small, more or less wedge-shaped, and surrounded by a peristomium which is larger than the prostomium. The peristomium contains the mouth ventrally and bears on the dorsum a pair of elongate, highly mobile palps which have a ciliated longitudinal groove. The parapodia are biramous with the lobes merely glandular cushions; ciliated branchiae are dorsal to the notopodia. In general, the setae are simple and, depending on the species, are either capillary-shaped (i.e., long and tapering) or hooked. Segments bearing setae are termed setigers.

The genus *Polydora* differs from other genera in the family by the presence of branchiae beginning posterior to a modified fifth setiger. A further distinguishing feature of this genus is that the fifth setiger bears setae modified into stout spines (see Figure G.1b).

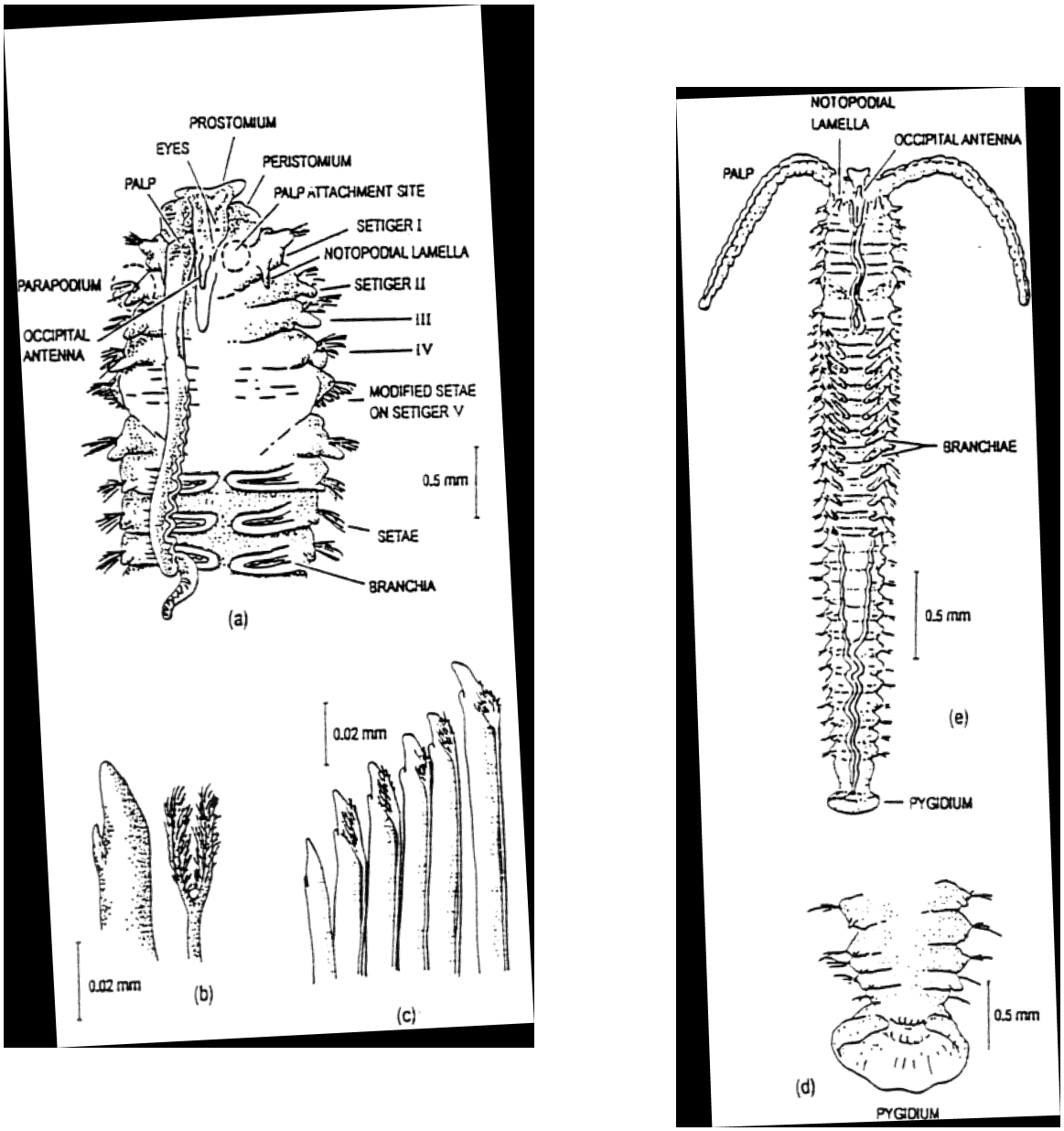

Figure G.1 Morphology of Juvenile and Adult Spionid Polychaete Worms, Including the Distinguishing Features of *Polydora cornuta* (Bosc, 1802)

[(a, with some modification), (b), (c), and (d) after Blake and Maciolek (1987); (e) after Hannerz (1956)].

(a) dorsal view of anterior end of adult member of Family Spionidae, showing diagnostic characters; (b) spine-like, modified setae of setiger 5; (c) arrangement of modified setae with their companion setae, for setiger 5 of *P. cornuta*; (d) pygidium of adult *P. cornuta*; (e) dorsal view of 33-setiger, young adult *P. cornuta*.

Polydora cornuta (Bosc,1802)

Figure G.1: Diagnosis

Diagnostic characteristics of the species include:

Prostomium. The prostomium is incised, flares laterally (almost appearing as two lobes), and extends posteriorly to setiger 3 as a caruncle. It bears an occipital antenna at the level of setiger 1. There are two pair of eyes; the anterior pair is larger and more widely spaced than the posterior pair. The peristomium is achaetous, contains a ventral mouth, and surrounds the prostomium, being visible dorsally. A pair of long palps project from the dorsum of the peristomium.

Body. Between the prostomium (head) and the pygidium (tail), each segment has a pair of laterally placed parapodia (feet) equipped with setae. Each segment so equipped is called a setiger. The upper lobes (notopodia) of the parapodia of setiger 1 bear notopodial lamellae subequal to the occipital antenna. Setiger 5 is larger than the other setigers and bears modified setae referred to as spines. Strap-like branchiae commence at setiger 7; they are dorsal to the notopodia, nearly full-sized at once, and reduced towards the posterior (Figures G.1a and G.1e). The pygidium is a conspicuous, saucer-shaped organ with a dorsal notch (Figure G.1d). It is lighter in colour than the flesh-coloured body.

Setae: Setae of anterior setigers are capillary-shaped. The modified spines of setiger 5 are falcate with a small tooth, and are accompanied by slender companion setae which have a delicate feathery end (Figure G.1d). The spines and companion setae are arranged in a row perpendicular to the body. Bidentate, neuropodial, hooded hooks (Figure G.1f) occur posterior to setiger 5.

GLOSSARY (after Fauchald, 1977)

Achaetous	- without setae (chaetae)
Biramous	- with two parts, as in a biramous parapodium
Branchiae	- gills
Capillary	- a word characterizing long, slender, tapering setae
Hook	- general term for stout, blunt, distally curved, dentate seta
Lamella (ae)	- parapodial process which might occur in front or behind lobe
Neuropodium	- ventral branch or ramus of a parapodium
Notopodium	- dorsal branch or ramus of a parapodium
Occipital	- pertaining to the postero-dorsal part of the prostomium
Palp	- sensory or feeding structure; often elongate
Parapodia	- paired, segmentally arranged structure extending from the body and usually bearing setae
Peristomium	- first distinct post-prostomial region, includes region of mouth
Prostomium	- anteriormost pre-segmental part of the body, often with eyes
Seta (ae)	- secretion from parapodia forming stiff structure (chaetae)
Setiger	- segment bearing setae (chaetae)

P. cornuta is typical of the family, and the adult is characterized by an elongated, subcylindrical body, tapering posteriorly and comprised of numerous short, similar segments (Figure G.1e). This species differs from other members of the genus in the shape of the modified setae on the fifth setiger (Figure G.1b), the shape of the pygidium (Figure G.1d), and the presence of a long, slender, occipital antenna equal in size to the paired notopodial lamellae of setiger 1 (Figure G.1a and G.1e). The occipital antenna along with the two notopodial lamellae on setiger 1 comprise the crown-like feature after which the animal was named.

P. ligni Webster (1879), a name which has been in the literature for many years, has now been designated a junior synonym of *P. cornuta* Bosc (1802) by Blake and Maciolek (1987). They redescribed the animal and designated a neotype from the original collection site. Throughout this report, however, the species is referred to as *P. cornuta* in keeping with its original identification and naming by Bosc in 1802.

Distribution and Ecology

P. cornuta occurs in Europe, where it has been reported from subtidal locations in the English Channel, the North, Baltic, and Mediterranean Seas, and off the coast of Denmark (Fauvel, 1927). In North America, it has been reported intertidally and subtidally from both the Atlantic and Pacific coasts and in the Gulf of Mexico (Blake and Maciolek, 1987). *P. cornuta* has a wide distribution on the Atlantic coast of North America, and has been reported from Labrador to South Carolina (Blake and Maciolek, 1987; Pocklington, 1989). In the Gulf of Mexico, it is a common inhabitant of coastal regions of Tampa Bay (Rice and Simon, 1980). On the Atlantic coast of Canada, this animal has been reported to be abundant intertidally in Nova Scotia on both the Atlantic coast (Conrad's Beach in Lawrencetown) and the Bay of Fundy coast (Minas Basin). *P. cornuta* has been found in numerous subtidal and intertidal locations for coastal waters of New Brunswick, both within the Bay of Fundy (L'Etang Estuary) and the Gulf of St. Lawrence (Miramichi River Estuary). There are additional records of *P. cornuta* from the St. Lawrence River Estuary, the north shore of Prince Edward Island, and the Northumberland Strait (Pocklington, 1989). The Pacific records of this species appear to be restricted to coastal areas of southern California (Rice, 1975), and might represent a recent introduction to those waters. Boat traffic[a] has often been cited as one of the causes of introduction of polychaetes and other aquatic invertebrate species to new geographic locations.

This species is a common inhabitant of estuaries of eastern North America, and tolerates low salinity. In eastern Canada, animals of this species can be collected from intertidal sandy-mud flats in the lower intertidal zone at Conrad's Beach, Lawrencetown, Nova Scotia. At this collection site, *P. cornuta* is abundant intertidally along with numerous other species of polychaetes and large numbers of *Macoma* sp. Salinity here varies between 8 and 28‰, as fresh water from a river-fed lake passes over the mud flats at low tide. Here, as elsewhere, large numbers of *P. cornuta* construct fragile tubes of silt on tidal flats that are exposed for a short time at low tide. The tubes extend up to 0.5 cm above the surface of the sediment and as much as 2 cm into the sediment. The animal captures silt particles from the water column for tube construction. It also deposits this silt to the seabed. *P. cornuta* is so efficient at silt deposition, that the species has been known to completely smother oyster beds with mud (Blake, 1969).

P. cornuta is a surface deposit feeder, picking up particles with its mobile palps and conveying them by means of ciliary action along the ciliated groove in the palp towards the opening of the tube. There, particles too large to be consumed are deposited or incorporated in tube construction. Food particles of the correct size are conveyed to the mouth. The animal aerates its body by circulating water through the tube and over the branchiae.

Reproduction and Development

Reproduction and development have been documented for this species. Fertilized eggs are produced in the presence of males, and reproduction is sexual (Rice and Simon, 1980). Blake (1969) and Rice (1975) both reported that several generations of *P. cornuta* can be produced per year[b], and that the larvae have a brief pelagic phase followed by their settlement to sediment and tube construction in the surficial (≤2 cm) layer.

Eggs are commonly seen in female *P. cornuta* that have reached a size of 33 setigers or more. There is a strong correlation between the number of egg capsules produced and the number of setigers of the adult worm. The eggs, which are found in setigers 14–21, appear grey to pink to orange in colour, and are laid approximately 4 to 5 days after they first appear in the coelom. They are deposited in egg capsules within the tube of sand and silt particles constructed by the female, and are attached to its inner walls by means of two adhesive threads

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[a] A most picturesque account of such exchange between Boston and the coast of California from 1837–39 is given in the book *Two Years Before the Mast* (Dana, 1911). Boat traffic might account for the introduction of *P. cornuta* to the Pacific coast of North America.

[b] Rice (1975) found that there were 13 spawnings of one individual worm over a four-month period.

(Hannerz, 1956). The capsules are aerated by ciliary action on the female's body, which produces a continuous flow of water through the tube. During development, egg capsules might contain numerous eggs as well as larvae at different stages of development. At a temperature of 22 ± 2°C, development of the eggs and larvae within capsules in the tube proceeds for approximately four days, at which time the female releases the larvae from the egg capsules and expels the larvae from the tube. Not all larvae are expelled from the tube at once. At time of release, the larvae have at least three setigers (Pocklington *et al.*, 1995). Figure G.2 illustrates the appearance of differing stages of larval development, for *P. cornuta*.

In the wild, the larvae of *P. cornuta* have been reported to be planktonic for approximately three weeks, during which time their development continues (see Figure G.2). Thereafter (i.e., after ~18 days of larval development), metamorphosis to a sediment-dwelling organism occurs (Blake, 1969). In the laboratory, the planktonic stage is completed more rapidly and metamorphosis to an infaunal, tube-dwelling life stage can begin when the animals are as young as one week post-emergence (i.e., one week after the larvae are released from the capsule and expelled from the tube). In a series of culturing trials and tests with *P. cornuta* held at 22 ± 2°C, Pocklington *et al.* (1995) found that adult characteristics such as long palps and cup-shaped pygidium could be observed within seven days of emergence, at which time (or sooner) the larvae settled and began tube construction. These studies determined that a complete (i.e., egg to egg) life cycle of *P. cornuta* could be completed within a period as short as 28 days under laboratory conditions. Animals used in tests were permitted to develop for two to three weeks' post-emergence (i.e., 2–3 weeks from the time that they were released from the capsule and expelled from the tube of the adult female), to ensure that an adequate number of similar-sized animals had fully metamorphosed, settled, and developed to the juvenile life stage.

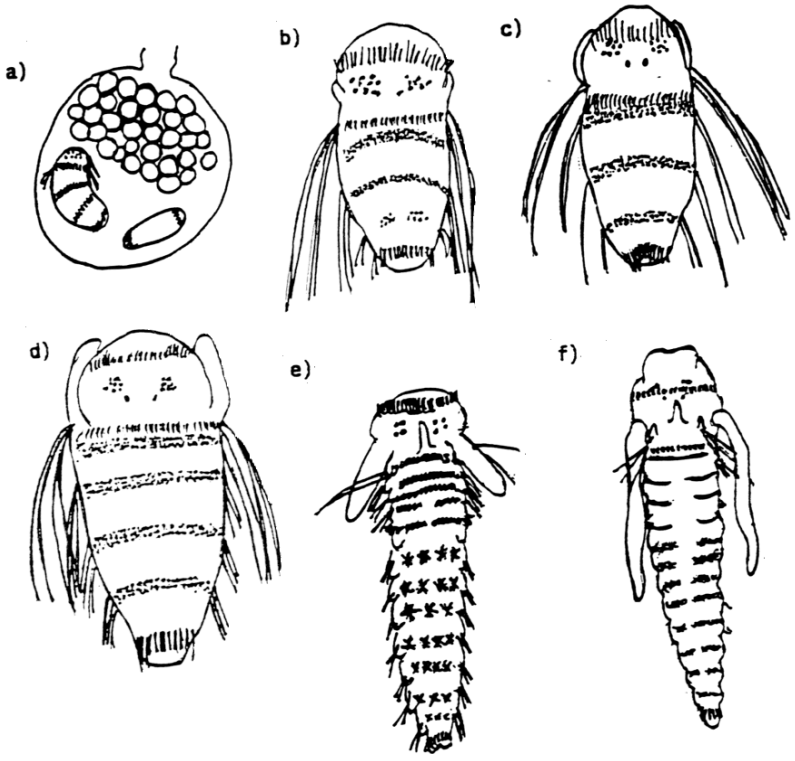

Figure G. 2 Morphology of Egg and Larval Developmental Stages of *Polydora cornuta* (Bosc, 1802)

(a) egg sac showing unfertilized eggs and larvae at different stages of development; (b) three-setiger larvae less than 24-hours old; (c) late three-setiger stage about 48-hours old; (d) four-setiger stage with protopalps discernible; (e) 12-setiger stage; (f) 16-setiger stage, part way through metamorphosis (note long palps and occipital antenna, both adult characteristics).

Recommended Procedures and Conditions Used by Environment Canada for Culturing the Spionid Polychaete Worm *Polydora cornuta* for Use in Sediment Toxicity Tests

H.1 Introduction

Environment Canada's Ocean Disposal Program identified a need for a chronic sublethal whole-sediment toxicity test using marine or estuarine polychaete worms. As a result of five years of research and a round-robin validation study, one spionid polychaete worm, *Polydora cornuta*, was chosen as the test species for this sediment toxicity test. The biological endpoints for this 14-day test are chronic survival and growth. This document outlines the procedures and conditions used by Environment Canada's Atlantic regional laboratory for culturing this species and raising same-aged juveniles for use in sediment toxicity tests.

H.2 Source of Animals

H.2.1 Field collections of *Polydora cornuta* can be obtained from Conrad's Beach, NS, or by contacting the ECB Toxicology Laboratory of Environment Canada, Atlantic Region, Moncton, NB at (506) 851-3486 or (506) 851-2907. Laboratory-cultured animals can also be obtained from this laboratory or from Environment Canada's Pacific Environmental Science Centre, North Vancouver, BC at (604) 924-2513 or (604) 924-2516.

H.2.2 Field-collected specimens are brought to the laboratory in sediment with overlying collection-site water. Acclimation to culture conditions should be gradual: $\leq 2^{o}$C per day; $\underline{\leq}$ 5 parts per thousand (‰) salinity per day.

H.3 Culturing *Polydora cornuta*

H.3.1 Culture temperature is 23 ± 2°C.

H.3.2 Photoperiod is 16h light:8h dark. The dark cycle normally runs from 2200 h to 0600 h, which is convenient for a normal laboratory work schedule, but the actual time of the dark cycle may vary from laboratory to laboratory. Lighting is by overhead full-spectrum tubes (fluorescent or equivalent, with a broad-spectrum wavelength), at an intensity which provides 500 to 1000 lux adjacent to the surface of the water within each culture chamber.

H.3.3 Culturing substrate is normally field-collected sediment from an uncontaminated site (e.g., Conrad's Beach, NS, where this species of spionid polychaete resides), which has been sieved through a 5-mm sieve and frozen at -20°C to kill polychaetes and other organisms present. Sediment is collected and separated into many small (e.g., 1.0 or 0.5 L) aliquots, which are placed in sealed plastic containers (e.g., Ziplok™ bags) that are then frozen. Quantities of sediment required are thawed as needed, and leftovers are discarded. Sediment is not refrozen for future use and should only be used for about 1 week after it is thawed. It must be stored at 4°C after thawing. Sediment should be washed several ($\geq$3) times with culture/test water before use. Alternatively, laboratory-formulated sediment having suitable grain-size characteristics may be used for culturing *P. cornuta*.

H.3.4 The salinity of the water used for culturing is normally 30 ± 4 ‰. These animals are euryhaline, and appear to be able to survive and grow normally when porewater salinity is as low as 15 ‰ salinity in sediment toxicity tests, without prior acclimation of the cultures and/or test organisms to lower salinity.

H.3.5 Feeding is normally three times per week. If the feeding schedule is altered (for example to twice a week, or to daily), the amount of food will have to be adjusted accordingly. Food is a 1:1 mixture (by weight) of Tetramarin™ fish food and oven dried (60°C) ground *Enteromorpha* (ET).[a] Seawater and ET are mixed and fed as a fine slurry. For example, 1 g of the dry, blended ET can be mixed with 20 mL of seawater using a mortar and pestle. Each mL will contain approximately 50 mg of ET. The slurry must be constantly mixed during feeding of the animals or settling out of the solids will occur. Feeding is usually supplemented by living cells of *Dunaliella tertiolecta*[b] cultured in Erdschreiber medium. This alga is available from the Department of Botany, University of Toronto, Toronto, ON (contact, Judy Acreman: jacreman@botany.utoronto.ca).

Erdschreiber medium can be made by adding the following ingredients to make up the desired amount of media:

- Stock A — Dissolve 20 g sodium nitrate ($NaNO_3$) in 1 L distilled water;
- Stock B — Dissolve 1.59 g anhydrous sodium phosphate (Na_2HPO_4) in 1 L distilled water;
- Stock C — Dissolve 0.15 mg Vitamin B_{12} in 1 L distilled water and freeze in 10-mL portions;
- Stock D — Soilwater (GR+) supernatant: add 5 teaspoons of garden soil (which has not been recently fertilized with commercial fertilizers and has a medium humus content) and a "pinch" of calcium carbonate to 1 L distilled water. Autoclave. While still hot, filter using a pre-filter (1.0 µm) to remove soil particles. Freeze in 50-mL portions;
- Stock E — PIV metal solution - Add 750 mg Na_2EDTA to 1 L distilled water and heat until dissolved (there will be a small amount of material not dissolved as the substance contains some insoluble material). Then add: 97 mg ferrous chloride ($FeCl_3 \cdot 4H_2O$), 41 mg manganese chloride ($MnCl_2 \cdot 4H_2O$), 5 mg zinc chloride ($ZnCl_2$), 2 mg cobaltous chloride ($CoCl_2 \cdot 6H_2O$), and 4 mg sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$). Mix until all ingredients have dissolved. Store at room temperature.

Choose the desired volume of Erdschreiber medium to make up, using Table H-1 below. Thaw out the required amount of Soilwater (GR+) and Vitamin B_{12}. Follow the table to make the desired amount of media. Thoroughly mix together in a clean container. Pour into clean flasks, fill about 1/3 full with media, plug with sponge stoppers, then cover with aluminum foil. Wrap the neck of the flask with a rubber band. Autoclave for 15 minutes at 121°C. The medium should be autoclaved on the day it is

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[a] The filamentous green alga *Enteromorpha* sp. (recognizable as 5–10 cm long emerald green algal filaments) is collected from rock pools at or above high tide along the shoreline at Peggy's Cove, NS (Zone 20, Easting 427000, Northing 4926600, Latitude-44°29'27", Longitude-63°55'05"). This alga is also available from British Columbia (contact G. van Aggelen, Toxicology Laboratory, Environment Canada, North Vancouver, BC, at 604-924-2513). The alga is scraped off the rock surfaces, for example with a spoon. Collections occur in June on the Atlantic coast, before mollusc populations heavily graze the alga. The *Enteromorpha* sp. is brought back to the laboratory, excess water is drained off, and the alga is blotted as dry as possible with paper towels and left on the laboratory bench on clean paper towels to dry further. Large debris (rocks, molluscs, and amphipods) are removed by hand-picking. As quickly as possible, thin layers (<1 cm thick) are placed in a drying oven at 60 °C. The dry *Enteromorpha* sp. (it is brittle) is coarsely ground using a mortar and pestle. It is stored in glass jars at 4 °C. When more food is required, a 1:1 mixture (by weight) of Tetramarin™ fish food and oven dried (60°C) ground *Enteromorpha* is finely ground in a Waring commercial blender at high speed, then press sieved through a 250 µm sieve. This mixture, known as ET, is stored at room temperature until required.

[b] *Dunaliella tertiolecta* is cultured in Erdschreiber medium under continuous light and continuous aeration at 25°C, for one week (7 to 9 days culture duration is acceptable) before use as food. When used as food, the concentration of this algal culture is approximately 3 to 4 million cells/mL, but the number of cells can vary substantially. For consistency, Environment Canada recommends that the percent light transmission at 540 nm wavelength of the algal culture be adjusted to 20 by dilution with seawater or concentration by low speed centrifugation at 2000 rpm for 15 minutes, and discarding the overlying water.

made up. Once autoclaved, cool immediately to reduce precipitate forming (a small amount of precipitate will remain). The media can be stored at room temperature indefinitely.

Table H-1 Preparation of Erdschreiber Medium

Ingredient	Volume of Media Required			
	1.0 L	**5.0 L**	**10.0 L**	**15.0 L**
Stock A	10 mL	50 mL	100 mL	150 mL
Stock B	10 mL	50 mL	100 mL	150 mL
Stock C	1 mL	5 mL	10 mL	15 mL
Stock D	50 mL	250 mL	500 mL	750 mL
Stock E	12 mL	60 mL	120 mL	180 mL
seawater	1 L	5 L	10 L	15 L

H.3.6 The feeding rate for *Polydora cornuta* varies with the age of the animals. *Polydora cornuta* releases planktonic larvae. Therefore, for approximately the first week until the larvae settle in the substrate, they can be expected to eat mainly the unicellular algae *Dunaliella*. Upon settling, the animals are called juveniles and the diet preference changes to the ET mixture.

Section H.3.7 provides a general outline of feeding rates; however, timing of ET feeding may vary. ET should be given as soon as settled animals are observed (this could be as early as three days following set-up), and amounts should be adjusted depending on numbers of settlers (estimated).

H.3.7 Feeding rates, at each feeding (assuming three feeding times per week) for *Polydora cornuta* are as follows:

Age	**Larval Stage**	**mL *Dunaliella* per 100 larvae/juveniles**	**mg ET per 100 larvae/juveniles**
Week 1	planktonic	25	0
Week 2	planktonic, some settle (benthic)	50	50
Week 3	most settled	25	100
≥ Week 4	settled juveniles/adults	25	200 (2mg/juvenile or adult worm)

H.3.8 Recommended culture chambers for *Polydora cornuta* are 2-L or 4-L food-grade plastic pails, each containing 20–25 g of collection-site sediment. These containers are used by Environment Canada's Atlantic regional laboratory as culture chambers for *P. cornuta,* since they are simple, inexpensive, and work well. They are used for broodstock animals (to produce young larval worms), as well as for raising juveniles for future broodstock or to be used as test animals. However, the chambers used could be any deep dish that is made of non-toxic material and available in the laboratory.

H.3.8.1 **Broodstock**: These animals are ≥3 weeks old (i.e., ≥3 weeks *post-release*). They are obtained from culture chambers that had been set up to raise and settle newly released larvae; see Section H.3.8.2, step (vi). They are removed from the substrate after three weeks culturing by gently prodding their tubes with a soft-bristled paintbrush, and they are then added to culturing chambers specifically set up

for broodstock along with 20–25 g of collection site sediment. Ten to twenty-five animals are added per culturing chamber for broodstock, depending on its size. Ten animals are commonly used if rearing broodstock in 2-L chambers, and 25 animals are commonly used in 4-L chambers.

The most common culturing chamber for broodstock is a 4-L food grade plastic pail, covered with a clean, clear acrylic (Plexiglass™) lid (20- to 25-cm square) with a 0.5-cm hole drilled in the centre. The lid slows evaporation and prevents large increases in culture salinity. A 1-mL glass pipette (cut at 10- to 12-cm long) attached to the end of airline tubing is used to aerate the culture. The hole size in the lid is such that the pipette slips through, but the airline is stopped at the lid. When cut at 10- to 12-cm long, it will not touch the bottom and injure the animals. The hole in the Plexiglass lid holds the pipette and prevents it from falling out whenever the culturing dish or airline is touched. Aeration is light and continuous. Excessive aeration could damage small worms and should be avoided.

The *Polydora cornuta* broodstock will produce young at high levels for over a month or so, but eventually will have to be replaced with younger animals. A reasonable practice is to begin growing planktonic larvae in 4-L pails after about two weeks to a month of larval production by the broodstock. After 3 weeks, the settled juveniles are ready to set up new broodstock dishes which will replace the older broodstock.

H.3.8.2 Planktonic larvae: At age 4 to 5 weeks and at a temperature of 23°C, the broodstock *Polydora cornuta* normally begin to release large numbers of planktonic larvae. Once these cultures are producing larvae, they should be renewed at least once a week (renewal frequency could be increased to twice a week when animals are to be cultured for testing purposes, to reduce the age and size variation among the test animals), and treated as follows:

(i) Aliquots of water from the broodstock culturing chamber (see Section H.3.8.1) are checked under a dissecting microscope for larvae.

(ii) If larvae are present in the water column, they are collected for counting. Since there might be large numbers of larvae (occasionally in excess of 4000 for each group of 25 broodstock animals), a simple procedure to estimate larval number is as follows:

- five or more 1-mL aliquots are taken from the broodstock culturing chamber, and the number of larvae per mL is estimated using a Sedgewick-Rafter counting cell observed under a dissecting microscope;
- calculate the mean number of larvae per mL;
- the number per mL is multiplied by the total volume of culture water in the broodstock culturing chamber to determine the total number of larvae in the chamber;
- record this number on the laboratory's bench sheet for cultures of *Polydora cornuta* (see Section H.4).

(iii) Replace water in the broodstock culturing chamber with new seawater previously aerated at 23 ± 2°C. Temporarily save the 'old' water from the culture containing the larvae in a clean 4-L food-grade plastic pail, if it is needed to set up new cultures to raise juveniles (see step vi).

(iv) Feed the broodstock worms. A slurry containing the ET (1:1) mixture is introduced to each broodstock culturing chamber along with a small volume of *Dunaliella tertiolecta* as per Section H.3.7 for ≥Week-4 animals.

(v) The lids and airlines are replaced on the broodstock culturing chamber, and aeration is started.

(vi) To culture and settle the larvae, and to raise juveniles, take a 2-L or 4-L food-grade polyethylene pail. Add 20–25 g of collection-site sediment to the pail. The volume of larvae-containing water (saved in the preceding step iii) equal to approximately 200 larvae (for a 2-L pail) or approximately

400 larvae (for a 4-L pail) is added to each pail. For example, if there was found to be an average of one larva per mL when counted, 400 mL of the larvae-containing water would be added to each 4-L pail, and 200 mL of this water would be added to each 2-L pail. Fill the pail with aerated culture water at 23 ± 2°C, and feed as per Section H.3.7 for Week-1 animals.

(vii) By removing the larval polychaetes twice weekly (for example, on Mondays and Thursdays), same-age larvae are obtained which are 0–4 days post-release (on Monday) and 0–3 days post-release (Thursday).

H.3.8.3 **Juveniles for toxicity tests**: The 2-L and 4-L pails (set up as in Section H.3.8.2, step vi) can be used to culture and settle the larvae and to raise juveniles for 3 weeks (actually 19–21 days after the pails are set up) for use as toxicity test organisms. It is important to raise approximately three or four times as many planktonic larvae as will be required for the toxicity test, since settling averages about 50% of the number of planktonic larvae but can vary considerably from pail to pail. To calculate the number of juveniles that will be required, multiply the number of sediments to be tested by 25 (5 replicates of 5 animals) and add 110 animals (60 animals for a reference toxicant test, plus 5 replicates of 5 control animals, plus 5 replicates of 5 animals for initial *time zero* weights). To summarize, calculate the number of juveniles required for the test, multiply by four, and settle this many larvae in 2-L or 4-L food-grade plastic pails, as described in Section H.3.8.2, step vi. Five to ten 4-L food-grade plastic pails with 25 broodstock adults per pail will usually provide enough larvae to start a sediment toxicity test.

H.4 Record Keeping

H.4.1 Maintenance of thorough culture records is essential for the generation of high quality test data. A generic laboratory bench sheet for cultures of *Polydora cornuta* is included at the end of this appendix to show the type of data that should be collected at each feeding and water-renewal period.

H.4.2 At each renewal/feeding period, temperature and salinity (‰) are measured in representative cultures and recorded on the bench sheet for cultures of *P. cornuta*. If these parameters are outside the recommended ranges of 23 ± 2°C and 30 ± 4 ‰ (Sections H.3.1 and H.3.4), corrective action should be taken, and the measured values recorded.

H.4.3 Amount and type of food should be recorded on the bench sheet for cultures of *P. cornuta*.

H.4.4 Other observations, such as number of young produced, culture water renewal, source of the animals in the culture, etc., should also be recorded.

Bench Sheet for Cultures of *Polydora cornuta*

Source of Animals: _____

Objectives for Culture: _____

Holding Conditions: _____

Identification of Culture Chamber(s): _____

Chamber Location(s):_____ **Date Started:** _____

No. of Original Animals/Culture Chamber: _____

Day-Specific Feeding Regime (3X/week), as follows:

Week 1: Dates_____ _____**mL *Dunaliella*** _____**mg 1:1 E:T**

Week 2: Dates_____ _____**mL *Dunaliella*** _____**mg 1:1 E:T**

Week 3: Dates_____ _____**mL *Dunaliella*** _____**mg 1:1 E:T**

Week 4: Dates_____ _____**mL *Dunaliella*** _____**mg 1:1 E:T**

Day-Specific Measurements and Observations						
Date	**Temp (°C)**	**Sal (‰)**	***Dunal* (mL)**	**E:T (mg)**	**No. Young**	**Observations and Comments**

Appendix I

Logarithmic Series of Concentrations Suitable for Toxicity Tests[a]

Column (Number of concentrations between 10.0 and 1.00, or between 1.00 and 0.10)[b]

1	2	3	4	5	6	7
10.0	10.0	10.0	10.0	10.0	10.0	10.0
3.2	4.6	5.6	6.3	6.8	7.2	7.5
1.00	2.2	3.2	4.0	4.6	5.2	5.6
0.32	1.00	1.8	2.5	3.2	3.7	4.2
0.10	0.46	1.00	1.6	2.2	2.7	3.2
	0.22	0.56	1.00	1.5	1.9	2.4
	0.10	0.32	0.63	1.00	1.4	1.8
		0.18	0.40	0.68	1.00	1.3
		0.10	0.25	0.46	0.72	1.00
			0.16	0.32	0.52	0.75
			0.10	0.22	0.37	0.56
				0.15	0.27	0.42
				0.10	0.19	0.32
					0.14	0.24
					0.10	0.18
						0.13
						0.10

[a] Modified from Rochinni *et al.* (1982).

[b] A series of five (or more) successive concentrations should be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by weight (e.g., mg/kg) or weight-to-volume (e.g., mg/L). As necessary, values can be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used, since such will provide poor resolution regarding the confidence limits surrounding any threshold-effect value calculated.