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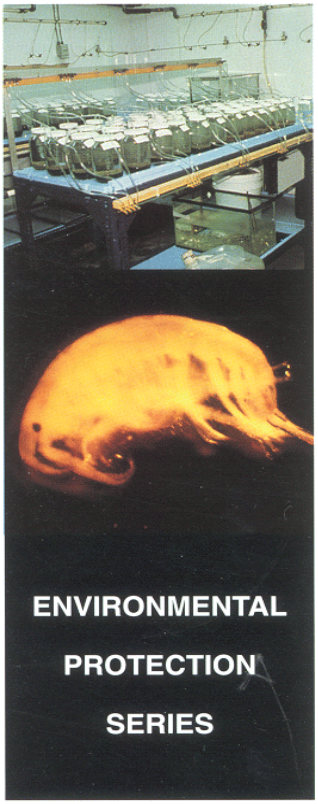

Biological Test Method: Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods

Environment Canada Environnement Canada

Canada

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Abstract

A reference method for measuring the acute lethal toxicity of contaminated whole sediment to marine or estuarine amphipods is described in this report. Explicit instructions are provided for performing a static, 10-day lethality test in the laboratory, using samples of estuarine or marine sediment and one or more of the following species of amphipod crustaceans: Rhepoxynius abronius, Eohaustorius washingtonianus, Eohaustorius estuarius, *and* Amphiporeia virginiana.

This reference method follows and is built upon the generic (multipurpose) biological test method "Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods" published previously by Environment Canada (1992; EPS 1/RM/26). It is intended for use with samples of contaminated marine or estuarine sediment.

Specific conditions and procedures are stipulated that include instructions on obtaining, shipping, holding, and acclimating test organisms; acceptable procedures and conditions for transporting, storing, and manipulating samples of sediment to be used in the test; required physicochemical analyses of sediment and water; procedures and conditions to be followed in preparing for and conducting the test; criteria for acceptable performance and valid test results; measurements and observations to be made; required or recommended data analyses; guidance for interpreting test results; and minimum reporting requirements. Instructions on the use of reference toxicity tests are also provided.

Résumé

Le présent rapport décrit une méthode de référence pour la mesure de la toxicité létale aiguë d'un sédiment entier contaminé pour les amphipodes marins ou estuariens. Il donne des instructions explicites pour l'exécution d'un essai d'une durée de 10 jours, en conditions statiques, au laboratoire, à l'aide d'échantillons de sédiment estuarien ou marin, en employant l'une ou plusieurs des espèces suivantes de crustacés amphipodes : Rhepoxynius abronius*,* Eohaustorius washingtonianus*,* Eohaustorius estuarius *et* Amphiporeia virginiana*.*

*La méthode de référence s'inspire de la méthode biologique générale (*Méthode d'essai biologique : essai de toxicité aiguë de sédiments chez des amphipodes marins ou estuariens*, publiée par Environnement Canada (1992 ; SPE 1/RM/26). Elle est destinée à l'examen d'échantillons de sédiments marins ou estuariens contaminés.*

Le rapport énonce les conditions et les modes opératoires précis qui doivent présider à l'obtention, à l'expédition, à la conservation et à l'acclimatation des organismes d'essai ; les conditions et les modes opératoires acceptables de transport, d'entreposage et de manipulation des échantillons de sédiment à utiliser dans l'essai ; les analyses physico-chimiques exigées pour le sédiment et l'eau ; les conditions et les modes opératoires à respecter au cours des préparatifs et de la réalisation de l'essai ; les critères d'acceptabilité de l'essai et de validité des résultats ; les mesures et observations à faire ; l'analyse nécessaire ou recommandée des données ; des orientations pour l'interprétation des résultats de l'essai ; les exigences minimales sur les rapports à produire. On y trouvera aussi des instructions sur l'emploi de toxiques de référence.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances or materials. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC aquatic toxicity laboratories;*
- *for testing that 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 management carried out by 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 life of specific test substances or materials destined for or within the aquatic environment. Depending on the biological test method chosen, substances or materials to be tested for toxicity could include samples of chemical or chemical substance, effluent, elutriate, leachate, receiving water or, where appropriate, sediment or similar particulate material.

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Terminology

The following definitions are given in the context of this report. Additional definitions in the detailed companion document (Environment Canada, 1992; including October 1998 Amendments) also apply here.

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 conditions such as temperature. The term usually refers to controlled laboratory conditions.

Compliance means in accordance with governmental permitting or regulatory requirements.

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

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

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.

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 test organisms.

Terms for Test Materials or Substances

Clean sediment is sediment that does not contain concentrations of any substance(s) causing discernible distress to the test organisms or their reduced 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/dilution water is the water used for preparing a series of concentrations of a test chemical, or that used as overlying water in a sediment toxicity test or as control water in a *water-only* test with a reference toxicant. Control/dilution water is frequently identical to the test (overlying) water.

Control sediment is *clean* sediment which does not contain concentrations of one or more contaminants that could affect the survival or behaviour of the test organisms. Control sediment might be natural sediment from an uncontaminated site, or formulated (reconstituted) sediment. This sediment must contain no added test material or substance, and must enable an acceptable survival rate for the test organisms during the test. The use of control sediment provides a basis for interpreting data derived from toxicity tests using test sediment(s).

Dredged material is sediment and/or settled particulate waste (e.g., solids from the sea bed of a harbour or channel) that has either been dredged from a waterbody or is being considered for dredging and subsequent ocean disposal.

Overlying water is water placed over sediment in a test chamber or holding/acclimation chamber.

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

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 contamination but within the general vicinity of the site(s) where samples of test sediment are collected.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order 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 at the time the test material 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 marine or estuarine amphipods is most often performed in the absence of sediment (i.e., as a *water only* test).

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

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.

Stock solution is a concentrated solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water to prepare the required 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 amphipods. In some instances, the term also applies to any solid-phase sample (including control sediment, reference sediment, or dredged material) 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 or for sieving the contents of each test chamber at the end of the test), and that used as control/dilution water for *water only* tests with 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.

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).

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

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, or 10-day LC50 for a sediment toxicity test with marine or estuarine amphipods).

Lethal means causing death by direct action. Death of amphipods is defined as the cessation of all visible signs of movement or other activity (e.g., a pleopod twitch) indicating life.

Lethality means causing death.

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

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

Toxicity test is a procedure for determining the effect of a material (e.g., dredged sediment) or substance (e.g., a reference toxicant) on a group of selected organisms of a single species (e.g., *Eohaustorius estuarius*), under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (*quantal;* e.g., % survival) or (b) the degree of effect shown (*graded* or *quantitative*; e.g., growth), after exposure to a specific test material or substance.

Acknowledgements

This reference method was written by D. McLeay (McLeay Environmental Ltd., Victoria, BC). The report is based on, and is a companion to, Environment Canada's generic (multipurpose) biological test method for measuring sediment toxicity using marine or estuarine amphipods (Environment Canada, 1992; EPS 1/RM/26), which includes but is not restricted to those species required for use herein.

R. Scroggins (Method Development and Application Section, Environmental Technology Centre, Environment Canada, Gloucester, ON) and J. Osborne (Marine Environment Division, Environment Canada, Hull, PQ) acted as Scientific Authorities and provided technical input and direction throughout the work. Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) participated in the development and review of this report and are thanked accordingly. Members of Environment Canada's regional and headquarters offices (Appendix B) are also thanked for their support.

Special acknowledgement is made of the many useful comments provided by each member of the Scientific Advisory Group responsible for scientific input and advice during the development and review phases related to the preparation of this report. This team of advisors included: Dr. P. Chapman (EVS Environment Consultants, North Vancouver, BC), Ms. C. Côté (Beak Consultants Ltée., Dorval, PQ), Mr. K. Doe (Environment Canada, Moncton, NB), Ms. M. Fennell (Environment Canada, North Vancouver, BC), Ms. C. Harris (Harris Industrial Testing Services Ltd., Hants County, NS), Ms. E. Jonczyk (Beak Consultants Ltd., Brampton, ON), Ms. D. Lee (B.C. Ministry of Environment, Lands and Parks, Surrey, BC), Ms. C. McPherson (EVS Environment Consultants, North Vancouver, BC), Ms. M. Murdoch (Jacques Whitford Environment Ltd., St. John's, NF), Ms. L. Porebski (Environment Canada, Hull, PQ), Mr. P. Riebel (P. Riebel and Associates, Baie-d'Urfé, PQ), Ms. J. Stewart (EVS Environment Consultants, North Vancouver, BC), Ms. D. Sullivan (Environment Canada, North Vancouver, BC), Dr. K.-L. Tay (Environment Canada, Dartmouth, NS), and Mr. G. van Aggelen (Environment Canada, North Vancouver, BC). Appendix C provides complete affiliations and points of contact for each member of this Scientific Advisory Group, and for the Scientific Authorities and Consultant.

Section 1

Introduction

This report specifies the procedures and conditions to be used according to this reference method, when preparing for and undertaking an acute (10-day) test for measuring the toxicity of samples of contaminated or potentially contaminated marine or estuarine sediment. The reference method herein is to be applied to one or more of the following four species of infaunal marine or estuarine amphipods: *Rhepoxynius abronius, Eohaustorius washingtonianus, Eohaustorius estuarius*, or *Amphiporeia virginiana*. This reference method represents one of the biological test methods to be used as part of sediment assessments consistent with the Federal *Ocean Dumping Regulations* under the *Canadian Environmental Protection Act* (EC, 1997).

Many components of the procedures and conditions specified herein are consistent with the guidelines and approaches for measuring sediment toxicity using marine or estuarine amphipods, as described in USEPA/USACE (1991), ASTM (1993), and USEPA (1994a). The contribution of those methods to all parts of this reference method is acknowledged, and they are recommended as sources of supporting rationale. Procedures stipulated in this report should, however, be taken as the definitive ones for regulatory purposes.

This reference method is compatible with the more detailed and complete guidance, instructions, and literature citations given in Environment Canada's multipurpose report EPS 1/RM/26 "*Biological Test Method: Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods"* (EC, 1992; including October 1998 Amendments). This biological test method (EC, 1992) is intended for use as a companion document when preparing for and undertaking the reference method described herein.

Sections 1.1 and 1.2 of Environment Canada (1992) should be consulted for further background information and for details regarding the historical use of the test. More recent references pertaining to the use of *R. abronius, E. washingtonianus, E. estuarius*, or *A. virginiana* in sediment toxicity tests are found herein in Appendices D, E, F, and G.

Section 2

Test Organisms

2.1 Choosing Species

One or more of the following species of marine or estuarine infaunal amphipods must be used with this reference method:

> *Rhepoxynius abronius,*
> *Eohaustorius washingtonianus*,
> *Eohaustorius estuarius*, or
> *Amphiporeia virginiana*.

Selection of one or more of these four species for use in a particular study must take into consideration the known or anticipated physicochemical characteristics of the test material (e.g., sediment grain size, porewater salinity, and porewater ammonia concentration) together with the known tolerance limits of the four candidate species to these characteristics. An investigator must be familiar with and/or consult the species-specific guidance provided in Appendices D (for *R. abronius*), E (for *E. washingtonianus*), F (for *E. estuarius*) and G (for *A. virginiana*) on the known tolerance and application limits for these four species, when choosing one to use in a test. In particular, the investigator should be aware that certain characteristics of each sample of test material to be evaluated using this reference method (namely, grain size and porewater salinity) must be within the species-specific application limits for these variables (see Section 2.6). Accordingly, the grain-size characteristics and porewater salinity of the test material needs to be known before choosing the test species. The investigator should also be aware of the known tolerance limits of each of these species to porewater ammonia (see Appendices D to G), and take this information into account together with that regarding known or anticipated concentrations of this contaminant in the test sediments, when choosing the test species and interpreting the findings of the test. Further guidance on the selection of test species is found in Section 2.1 of Environment Canada (1992).

2.2 Life Stage, Size, and Source

Juvenile or adult animals representing each of these species, which measure 3 to 5 mm total length, are available year round (EC, 1992) and should be used for this test. Very large individuals (i.e., those >5 mm total length) must not be used. Organisms that are too small to be retained on a 0.5-mm mesh screen should not be used.

All amphipods used in a test must be derived from the same population and source. In Environment Canada (1992), Appendices E (for *A. virginiana*), G (for *E. estuarius*), H (for *E. washingtonianus*), and K (for *R. abronius*) provide guidance on the appearance, behaviour, and distribution (including possible collection sites) of the species of amphipods to be used with this reference method. Sources commonly used for collection of test organisms include: West Beach, Whidbey Island, WA for *R. abronius*; Witty's Lagoon or the exposed side of Esquimalt Lagoon, Victoria, BC for *E. washingtonianus*; Beaver Creek, Newport, OR for *E. estuarius*; and Martinique Beach, Halifax County, NS for

A. virginiana. There are commercial vendors experienced in collecting and shipping these species. Laboratory personnel should be confident that any person(s) undertaking the collection, handling, and transport of organisms to be used with this reference method is/are fully familiar with and follow(s) recognized practices in these respects (EC, 1992; USEPA, 1994a), and that the organisms provided are indeed the correct species. For further information, contact Environment Canada or other members of the Scientific Advisory Group (Appendix C).

2.3 Collection, Handling, and Transport

Guidance given in Section 2.4 of Environment Canada (1992) should be followed when collecting, handling, and transporting amphipods. It is critical that standard, proven procedures be used to ensure that consistently healthy animals are obtained for the toxicity tests.

Containers used to transport amphipods are usually those used to hold and acclimate the organisms at the laboratory. Suitable containers with sealable lids include plastic food containers or plastic pails. At the collection site, a minimum 2- to 4-cm (or thicker) layer of sieved (0.5- to 1.0-mm mesh screen) sediment from the place where the animals are collected should be placed in the bottom of the container. Water from the collection site is then added to form a layer of $\geq$2 cm of overlying water. Amphipods sieved from other aliquots of the collection site sediment should then be transferred gently to the container. The density of amphipods in the container should not exceed 1 amphipod/cm^2 (USEPA, 1994a).

An appropriate quantity of sediment should be collected, sieved, and transported with the animals, for use as control sediment in the sediment toxicity test.

Long-distance transport (i.e., by air shipment) of *E. washingtonianus* or *A. virginiana* is not recommended because of unacceptably high mortality during holding and acclimation, or in control sediment during the test. An investigator may choose to do so rather than using an alternate species (i.e., *R. abronius* or *E. estuarius*); however, the species-specific criterion for a valid test (Section 4.6) defined herein must be met if the results of the test are to be considered valid and acceptable according to this reference method.

2.4 Holding and Acclimation

Guidance provided in Section 2.5 of Environment Canada (1992) should be followed when holding and acclimating each group of amphipods that has been collected for use in a sediment toxicity test.

Field-collected animals must be acclimated to the lighting, temperature, and salinity (for water overlying sediment) conditions to be applied during the test (see Section 4), for a minimum of two days and a maximum of ten days before the start of the test. Additionally, animals should not be held at the test facility for more than ten days following their collection, before the test is initiated.

Upon receipt of field-collected animals at the laboratory, the quality (i.e., temperature, salinity, dissolved oxygen, and pH) of the overlying water in one or more of the

containers holding field-collected animals and sediment from the collection site should be determined and recorded. Any dead organisms observed on the surface of the sediment should be counted and removed, together with any debris evident. Sieving of the sediment in the container at any time before the day that the test is started is not recommended, since this procedure could unduly stress the test organisms. To minimize disturbance, amphipods should be held and acclimated in the container(s) used to collect and transport them. Alternatively, the organisms and sediment within the collection container(s) could be transferred (without sieving) to a larger holding/acclimation chamber if considered necessary to reduce crowding and increase surface area.

During the holding and acclimation period, amphipods should be held unfed in a minimum 2- to 4- cm (or deeper) layer of sediment from the collection site. Water overlying this sediment should be at least 2 cm deep. The dissolved oxygen content of this water must be maintained at 90 to 100% saturation by aeration or, if applied, by continuous replacement with oxygen-saturated water. Depending on the duration of the holding/acclimation period, the overlying water should be replaced continuously or periodically (e.g., daily) with air-saturated, fresh seawater adjusted to the required temperature and salinity.

During the holding and acclimation period, lighting must be constant and continuous. Overhead broad-spectrum (fluorescent or equivalent) lights should be used. Light intensity adjacent to the surface of the overlying water in the holding/acclimation container(s) should be 500 to 1000 lux.

The temperature of the overlying water must be adjusted gradually (i.e., no more than 3°C change per day) to a daily-mean acclimation temperature of 15 ± 2°C if acclimating *R. abronius, E. washingtonianus,* or *E. estuarius*; and to 10 ± 2°C if acclimating *A. virginiana*. Thereafter, amphipods must be held at the same respective temperature for a minimum of two days before their use in a test.

The salinity of the overlying water must be adjusted gradually (i.e., no more than 5‰ change per day) to a value representative of that of the porewater salinity measured for the sample(s) of test material. Thereafter, amphipods must be held at this salinity for a minimum of two days before their use in a test. In instances where a number of samples of test material (e.g., from different sampling stations and/or depths) are to be tested concurrently, the salinity of the overlying water must be adjusted to the mean porewater salinity determined for these samples (Section 4.3). The salinity to which test organisms are acclimated must be within their (species-specific) application limits (see Section 2.6). To minimize the interval between collection of test organisms and the start of the test, the necessary salinity and temperature adjustments (to acclimation and test conditions) may be conducted concurrently.

The temperature and salinity of the overlying water in each holding/acclimation chamber should be measured at least daily during any initial period of adjustment. Thereafter, the temperature, salinity, pH, and dissolved oxygen concentration in the overlying water must be measured at the beginning and end of the remaining period of acclimation (i.e., 2 to 10 days), as a

minimum. It is recommended that temperature and salinity be measured daily during this period.

Water used to hold and acclimate test organisms may be that from an uncontaminated supply of natural seawater or reconstituted seawater. Guidance provided in Section 2.5.4 of Environment Canada (1992) should be consulted and followed when preparing and storing this water, and for monitoring its quality.

2.5 Selection of Test Organisms

The appearance and behaviour of amphipods in each holding/acclimation container should be "normal" and typical of the species (see EC, 1992). Any animal that fails to burrow in the holding sediment, or that appears or behaves atypically during the holding/acclimation period, must be discarded. Additionally, any animals that appear or behave atypically (see Section 2.2) when they are sieved from the collection-site sediment on the day that the test is started (Section 4.7) must be discarded. Records should be kept of the number of amphipods seen on the surface of the sediment or in the overlying water during the holding and acclimation period. The number of dead or atypical animals removed from each holding/acclimation container should also be recorded, for each period of observation.

2.6 Species-specific Application Limits

The physicochemical characteristics of each test sediment must be known before the species of test organism is selected. The choice of amphipod species to be used in a particular sediment toxicity test depends on the porewater salinity and grain size characteristics of the test material. The species-specific application limits in Table 1 must be followed, when choosing the test species as well as when acclimating them and undertaking the sediment toxicity test according to this reference method (see Appendices D, E, F, and G for details).

Table 1 Species-specific Application Limits for Reference Method

Test Species	Acceptable Physicochemical Characteristics of Test Sediment			
		sediment grain size		
	porewater salinity (‰)	percent very coarse-grained [a]	percent fines [b]	percent clay [c]
Rhepoxynius abronius	must be 25 to 35	0 to 100 is acceptable	must be <90	must be <40
Eohaustorius washingtonianus	must be 15 to 35	must be <25	must be <80	must be <20
Eohaustorius estuarius	must be 2 to 35	must be <90	0 to 100 is acceptable	must be <70
Amphiporeia virginiana	must be 15 to 35	0 to 100 is acceptable	must be <90	must be <35

[a] Percentage of particles in test material >1.0 mm in size.

[b] Percentage of particles in test material <0.063 mm (i.e., % silt and clay) in size.

[c] Percentage of particles in test material <0.004 mm in size.

Section 3

Facilities, Equipment, and Supplies

Facilities used to hold and acclimate amphipods, and to undertake toxicity tests, must be well ventilated, free of fumes, and isolated from physical disturbances or airborne contaminants that might affect the test organisms. The testing facility should be isolated from the area where amphipods are being held and acclimated to test conditions. The holding/acclimation and testing facilities should also be isolated from areas where test sediments or stock solutions of chemicals are prepared, and removed from areas where equipment is cleaned.

The separate facilities where organisms are acclimated and tests are performed must enable the temperature of the water overlying sediment to be held within the desired range (i.e., 15 ± 2°C for *R. abronius, E. washingtonianus,* or *E. estuarius*; and 10 ± 2°C for *A. virginiana*). This may be achieved using environmental chambers, temperature-controlled recirculating water baths, or equivalent facilities with rigorous temperature control. Overhead lighting by fluorescent or equivalent broad-spectrum illumination should provide a light intensity of 500 to 1000 lux adjacent to the surface of the overlying water, in both the holding/acclimation and testing facilities.

Equipment and supplies which contact sediments, water, or stock solutions must not contain substances that can be leached or dissolved in amounts that adversely affect the test organisms, and should be chosen carefully to minimize sorption of materials from water. Guidance provided in Section 2.5.2 of Environment Canada (1992) should be followed when choosing equipment and supplies.

High-density plastic containers are recommended for holding and acclimating amphipods. Glass containers (beakers or wide-mouthed jars) with a capacity of approximately 1 L and an internal diameter of approximately 10 cm, together with suitable covers (e.g., watch glasses or plastic lids) must be used as test chambers. All test chambers and other equipment that come in contact with sediment, water, or test organisms must be cleaned, and rinsed just before use (see guidance in Section 3.3 of Environment Canada, 1992).

Section 4

Procedure for Testing Sediment

4.1 Sample Collection

Guidance on the collection of samples of marine or estuarine sediment for toxicity evaluations using marine or estuarine amphipods is given in Section 5.1 of Environment Canada (1992), and should be consulted beforehand. Environment Canada (1994) provides additional guidance on field sampling designs and appropriate techniques for sample collection; this guidance document should be referred to for further information.

Procedures and equipment used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives or regulatory requirements, and on the nature of the material being sampled. Samples of dredged material should be taken at all depths of interest. Samples of field-collected test or reference sediment, including those taken from or adjacent to ocean disposal sites, frequently represent the upper 2-cm depth. Sites for collecting samples of reference sediment should be sought where the geochemical properties of the sediment, including grain size characteristics, are similar to those at the site(s) where samples of test sediment are collected. Ideally, reference sediment should be collected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the site(s) where samples of test sediment are taken. It is recommended that reference sediment from more than one site be collected to increase the likelihood of a good match with grain size and other physicochemical characteristics of the test sediments.

Samples of control sediment are normally those taken at the site where test organisms are collected.

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. Additional guidance on sampling is found in Environment Canada (1995) for disposal-at-sea applications. Applicants are encouraged to consult with their regional Environment Canada Ocean Disposal Office (see Appendices B and C for contact information), before sampling and testing.

Where practical and consistent with the study design and objectives, a minimum of five samples of sediment should be taken from each discrete sampling station and depth of interest. Where practical and appropriate (see Section 6), sample collection should also include ≥ 5 samples from each of one or more reference stations (i.e., sites where uncontaminated sediment, having physicochemical properties similar to that of the test sediments, can be found) within the vicinity. The objective of collecting replicate samples at each station is to allow for quantitative statistical comparisons within and among different stations (EC, 1994; 1998a). Each of these "true replicate" samples of sediment should

be tested for its acute toxicity to amphipods, using a minimum of five test chambers per sample (i.e., laboratory replicates) (EC, 1992).

The collection of replicate samples at a given sampling station is often not necessary for certain dredging projects (EC, 1994). If the objective is to obtain a "cost-effective" assessment of sample toxicity within the project area, sampling as many stations as possible (subject to cost constraints) with a single sample from each station might be the best way to achieve this. In this instance, testing might be restricted to five laboratory replicates (i.e., 5 subsamples) per sample (and no replication of samples from each station), each of which is prepared in the laboratory (Section 4.3).

To sample sediment, a benthic grab (i.e., Smith-MacIntyre, Van Veen, PONAR) or core sampler should be used rather than a dredge, to minimize disruption of the sample. Care must be taken during sampling to minimize loss of fines. The same collection procedure should be used for all field sites sampled.

A per-sample volume of at least 5 to 7 L of whole sediment is frequently required (EC, 1994), although this will depend on the study objectives/design and on the nature of the physicochemical 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.

4.2 Sample Labelling, Transport, and Storage

Instructions and guidance in Section 5.2 of Environment Canada (1992) pertaining to sample labelling, transport, and storage apply here, and should be reviewed and followed. Additional useful guidance in this respect is found in Environment Canada (1994) and USEPA (1994a).

Containers for transporting and storing samples must be new or thoroughly cleaned, and rinsed with clean water. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. 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 which can be used to identify the sample or subsample. A cross-referenced record, which might or might not accompany the sample or subsample, must be made by the field personnel identifying the sample type (e.g., grab, core, composite), source, precise location (e.g., water body, latitude, longitude, depth), replicate number, and date of collection. This record should also include the name and signature of the sampler(s). Sediment sample collectors should also keep records describing:

- the nature, appearance, volume and/or weight 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 the 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 transport (EC, 1994). As necessary, gel packs, regular ice, or other means of refrigeration should be used to assure that sample temperatures range within 1 to 7°C during transit.

Upon arrival at the laboratory, the sample temperature and date of receipt must be recorded. Samples to be stored for future use must be held in airtight containers and in darkness at 4 ± 2°C (EC, 1992; 1994). 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, 1992; 1994). 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.

4.3 Sample Manipulation and Characterization

Samples of field-collected test sediment and reference sediment must not be wet-sieved. Large debris or large indigenous macro-organisms should be removed using forceps or a gloved hand. If a sample contains a large number of indigenous macro-organisms which cannot be removed using forceps or a gloved hand, the sample may be press-sieved (not washed) through one or more suitably sized (e.g., 1 or 2 mm) mesh stainless steel screens. Any pore water that has separated from the sample during shipment and storage must be mixed back into the sediment. To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample should normally be stirred using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture and colour are homogeneous (EC, 1992). Alternatively, a mechanical method (USEPA, 1994a; EC, 1994) 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.

The portion of control sediment obtained from the amphipod collection site for use in the toxicity test, and for particle size and chemical analysis, must be previously wet-sieved through a 0.5-mm stainless steel screen to remove small amphipods and other organisms. Procedures described in Section 3.4 of Environment Canada (1992) should be followed. Sieved control sediment should be stored as described in the previous section (4.2) until used.

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, and in the labelled containers required for storage of samples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using amphipods or other test

organisms should also be transferred at this time to labelled containers. 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 until used or analyzed. Just before it is analyzed or used in the toxicity test, each subsample must be thoroughly re-mixed to ensure that it is homogeneous.

Each sample (including all samples of control and reference sediment) must be characterized by analyzing subsamples for at least the following (EC, 1992; USEPA, 1994a): for whole sediment — percent very coarse-grained sediment (i.e., particles >1.0 mm), percent sand (>0.063 to 2.0 mm), percent silt (>0.004 to 0.063 mm), percent clay (<0.004 mm), percent water content, and total organic carbon content; for pore water — salinity, pH, and ammonia (total and un-ionized). Other analyses could include: total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and porewater analyses for various physicochemical characteristics such as hydrogen sulphide. Recommended procedures for collecting pore water are described in Environment Canada (1994) and should be followed here. For disposal-at-sea applications, minimum information requirements are explained in Environment Canada (1995).

Analyses for particle size distribution and porewater salinity must be undertaken as soon as possible after sample collection, to confirm that the values for these characteristics are within the application limits for the intended species of test organism (see Section 2.6 as well as Appendices D for *R. abronius*, E for *E. washingtonianus*, F for *E. estuarius*, and G for *A. virginiana*). Analyses for porewater pH, salinity, and ammonia must be undertaken within 24 h of the start of the test and should be initiated at the beginning of the test, to determine the initial concentrations of total and un-ionized ammonia to which test organisms were exposed at the start of the test. Ammonia analyses must be conducted using a recognized and standardized procedure (for example, APHA *et al.*, 1995; Standard Methods). Calculations of concentrations of un-ionized ammonia must be based on the test temperature and on the porewater pH and salinity of the sample (Trussell, 1972; Bower and Bidwell, 1978; USEPA, 1985).

4.4 Test Water

Test water must be the same as that used to acclimate the test organisms (see Section 2.4). This may be reconstituted seawater or an uncontaminated supply of natural seawater. Natural or reconstituted seawater may be adjusted to the required salinity (i.e., that to which the amphipods have been acclimated; see Section 2.4) by the addition of dry ocean salts or brine (if too brackish), or distilled water (if too saline). Guidance provided in Environment Canada (1992; Section 2.5.4) for preparing and storing test water should be followed.

Test water must be adjusted to the required test temperature (i.e., 15 ± 2°C for *R. abronius, E. washingtonianus*, or *E. estuarius*; 10 ± 2°C for *A. virginiana*) and salinity before use, and its dissolved oxygen concentration must be 90 to 100% of the air-saturation value for that temperature and salinity. As necessary, the required volume of water should be aerated vigorously (using oil-free compressed air passed through one or more air stones) immediately before use,

and its dissolved oxygen content checked to confirm that 90 to 100% saturation has been achieved.

4.5 Test Conditions

- This is a static, whole sediment toxicity test, during which the overlying water is not renewed.
- Test duration is 10 days.
- For *R. abronius, E. washingtonianus*, and *E. estuarius*, the test must be conducted at a daily mean temperature (overlying water) of 15 ± 2°C. Additionally, the instantaneous temperature must be 15 ± 3°C at all times during the test.
  For *A. virginiana*, mean and instantaneous temperatures must be 10 ± 2°C and 10 ± 3°C, respectively.
- At the start of the test, the salinity of the overlying water must be the same as that to which the test organisms have been acclimated (see Section 2.4).
- Sediment in each ~1-L test chamber must be present as a uniform, 175-mL layer, approximately 2 cm thick, with a 775-mL layer of overlying water.
- Each test chamber must be covered.
- Overlying water in each test chamber must be aerated continuously at a gentle rate which does not cause turbulence or disturb the surface of the sediment. This rate should maintain a DO concentration in the overlying water of ≥90% saturation, throughout the test.
- Lights must be left on continuously throughout the test. Intensity adjacent to the surface of the overlying water should be 500 to 1000 lux.
- Test organisms must not be fed during the 10-day test period.

4.6 Criteria for a Valid Test

- For *R. abronius* or *E. estuarius*, the mean 10-day survival rate in control sediment must be at least 90%.
- For *E. washingtonianus*, the mean 10-day survival rate in control sediment must be at least 85%.
- For *A. virginiana*, the mean 10-day survival rate in control sediment must be at least 80%.
- Results for a particular test sediment (including reference and control sediment) are valid only if its grain size characteristics and porewater salinity are within the application limits specified for the species of test organism used (see Section 2.6).

4.7 Beginning the Test

Details for preparing for and starting the test are provided in Section 4.1 of Environment Canada (1992); instructions therein should be followed when undertaking this reference method.

Each test chamber placed within the test facility must be clearly coded or labelled to enable sample identification. The date and time when the test is started must be recorded, either directly on the labels or on separate data sheets specific to the test. The test chambers should be positioned for ease of observation and taking measurements. A minimum of five replicates per treatment,

including at least five samples or subsamples of control sediment, should be included in each test (see Section 4.1). Each set of replicate treatments should be positioned randomly within the test facility.

On the day preceding the start of the test (i.e., Day -1) each sample of test sediment to be evaluated should be homogenized (Section 4.3). Thereafter, a 175-mL aliquot of each sample or subsample must be added to a separate test chamber. The aliquot should be smoothed to form a layer approximately 2-cm deep on the bottom of the test chamber, either by tapping the side of the test chamber against the side of the hand or by smoothing the sample with a clean plastic or stainless steel spatula. Highly contaminated sediment should be added to test chambers in a certified fume hood. Following sample addition, test water (see Section 4.4) should be added without disturbing the sample (see EC, 1992; Section 4.1), to a standard height on the test chamber. The (identical) volume of water added to each test chamber should approach the 950-mL mark (i.e., the combined volume of sediment and overlying water in the chamber at the start of test), but allow space for the transfer of test organisms (in a small volume of test water) the next day (i.e., Day 0). Each test chamber should then be covered, placed within the temperature-controlled test facility, and the overlying water aerated gently. Sediment in test chambers must not be stirred with the overlying water or otherwise disturbed, at any time before (i.e., Day -1) or during the test.

The overlying water in each test chamber must be aerated continuously once the water is added (i.e., Days -1 through Day 10); except perhaps during the brief period when test organisms are added, and when observations and measurements are made during the test. Compressed air, previously filtered and free of oil, should be bubbled through a glass or plastic pipette and attached plastic tubing (aquarium supply). The tip of the pipette should be suspended 2 to 4 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle (e.g., 2 to 3 bubbles/s), and should not disturb the surface of the sediment. The rate of air flow should be adjusted as required to maintain a dissolved oxygen concentration in the overlying water of at least 90% saturation (EC, 1992; USEPA, 1994a).

Instructions in Section 4.1 of Environment Canada (1992) should be followed when adding amphipods to test chambers the next day (i.e., Day 0). Test organisms must be sieved from their acclimation chamber(s) (see Sections 2.4 and 2.5, herein) on that day, and 20 amphipods randomly added to each test chamber. Any animal that appears atypical or that is dropped or injured during the sieving and transfer process must not be used. Following the addition of test organisms, the water level in the test chamber must then be brought up to the 950-mL mark, after which the test chamber is covered and aeration of the overlying water is resumed after one hour at a gentle rate.

Within the first hour of the test, each test chamber must be examined to see if the amphipods have buried into the sediment. With the exception of *A. virginiana* (see EC, 1992; footnote 18), animals that do not bury within one hour must be replaced with those from the same sieved population, unless they are observed to repeatedly burrow into the sample and immediately emerge in an apparent avoidance response, or unless there

is an obvious difference between the control and the test sediments. This would indicate a contaminant-related response, in which instance animals in any treatment would not be replaced. Amphipods displaying an avoidance behaviour during the initial hour of the test must not be replaced, i.e., they are to comprise the 20 test animals in the test chamber. Observations of apparent avoidance responses must be recorded.

4.8 Test Measurements and Observations

Test measurements must be made in at least one test chamber representing each treatment. The temperature of the overlying water must be measured at the beginning of the test and thereafter at least three times per week (e.g., Mondays, Wednesdays, Fridays) on non-consecutive days until test completion. More frequent (i.e., daily) measurements of temperature 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, and thereafter at least three times/week (e.g., Mondays, Wednesdays, Fridays) on non-consecutive days until test completion. More frequent (e.g., daily; USEPA, 1994a) measurements are recommended and should be performed for sediments having a high oxygen demand that depresses the dissolved oxygen of the overlying water below 90% saturation. A probe and calibrated dissolved oxygen (DO) meter is recommended for these measurements. The probe must be inspected carefully after each reading to ensure that organisms are not adhered to it, and must be rinsed in deionized or distilled water between samples to minimize cross-contamination. The position of the tip of the pipette in each test chamber and the rate of aeration should be checked frequently and routinely (e.g., daily) throughout the test, and adjustments made if necessary to maintain a gentle (e.g., 2 to 3 bubbles/s) rate of aeration.

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 re-established at a gentle rate. Any DO readings that have fallen below 60% saturation (USEPA, 1994a) must be included in the test-specific report (Section 7.1.6), and must be considered when interpreting the test results (Section 6.2).

The salinity and pH of the overlying water must be measured at the beginning and end of the test in at least one test chamber representing each treatment. Additionally, ammonia concentrations in the overlying water must be measured (total ammonia; see for example APHA *et al.*, 1995) and calculated (un-ionized ammonia; Trussell, 1972; Bower and Bidwell, 1978; USEPA, 1985) at the beginning and end of the test in at least one test chamber representing each treatment. Salinity and pH may be measured using probes and calibrated meters. 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, samples of

overlying water must be taken just before the addition of test organisms and upon completion of the test. On each occasion, no more than 10% of the volume of the overlying water in a test chamber should be removed for this purpose. A pipette should be used carefully to remove water from a depth of about 1 to 2 cm above the sediment surface. The pipette should be checked to ensure that no amphipods are removed during water sample collection.

Each test chamber must be examined frequently and routinely during the test (i.e., at least three times per week on non-consecutive days, and preferably daily) to note if amphipods have emerged from the sediment, or if they are swimming in the overlying water or floating on its surface. The number of animals seen swimming in the water, floating on its surface, moving on the surface of the sediment, or emerged from the sediment but apparently dead, should be noted and recorded during each observation period. Amphipods caught in the surface film should be gently pushed down into the water using a glass rod or pipette. Animals that appear to be dead should not be removed.

4.9 Ending the Test

The test is terminated after 10 days of exposure. At that time, the final set of observations of numbers of amphipods seen floating on the surface of the overlying water, swimming in it, moving on the surface of the sediment, or emerged from the sediment but apparently dead, must be made and recorded. Just before sieving the contents of a test chamber, all live and apparently dead amphipods in the water column or on the surface of the sediment should be pipetted from the test chamber. Individuals that 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 pleopod twitch). Any animals that fail to show signs of life before and after prodding must be counted as dead.

A consistent amount of time should be taken to sieve the contents of each test chamber for recovery of live or dead organisms. To ensure that the procedure used to recover amphipods is adequate, it is recommended that the laboratory personnel responsible for sieving the contents of test chambers demonstrate that they are able to retrieve an average of at least 90% of the organisms from control sediment. For example, test organisms could be added to control sediment and recovery could be determined after one hour (USEPA, 1994a; Tomasovic *et al*., 1995).

The contents of each test chamber must be sieved through a 1.0-mm (or smaller) mesh screen to remove all remaining test organisms, and to determine if they are dead or alive. Test water adjusted to the salinity and temperature of that in the test chambers should be used for sieving. Material retained on the screen should be washed into a sorting tray using clean test water. A small portion of the material should be sorted through at a time, removing amphipods as they are found (USEPA, 1994a). Amphipods that are inactive but are not obviously dead should be examined closely as described previously, and counted as dead if they fail to show signs of life. Animals

that are missing are presumed to have died and are counted as dead organisms in the calculations (Section 4.10).

4.10 Test Endpoints and Calculations

The biological endpoint for this 10-day solid-phase sediment toxicity test is percent survival. The mean (± SD) percentage of amphipods that survived the 10-day exposure is calculated, for each treatment (i.e., each set of replicates representing a test sediment). This calculation is typically based on 100 organisms per treatment (i.e., 20 amphipods exposed to each of five replicate samples or subsamples; see Sections 4.1 and 4.7).

To enable this calculation, numbers of amphipods found to be dead, missing, or alive in each test chamber at Day 10 are determined and recorded (Section 4.9). Missing individuals are assumed to have died and disintegrated during the test, and must be included in the count of number dead per chamber. The mean (± SD) percent survival for the replicate groups within a given treatment is then calculated, for each treatment. Thereafter, the mean (± SD) value for percent survival determined for each treatment is compared against that for the reference sediment or, as necessary, against the mean percent survival for the control sediment (see Section 6 for guidance).

Section 5

Procedure for Testing a Reference Toxicant

The routine use of a reference toxicant is necessary to assess the relative sensitivity of the populations of amphipods used, and the precision and reliability of data produced by the laboratory personnel for that reference toxicant, under standardized test conditions. When determining the toxicity of samples of marine or estuarine sediment to marine or estuarine amphipods according to this reference method, a static, *water-only* reference toxicity test must be performed on each batch of field-collected organisms used for testing. A guidance document on controlling the precision of toxicity tests using water-only reference toxicity tests has been published by Environment Canada (1990), and provides useful background information and instructions in this respect.

The reference toxicity test to be conducted with each batch of field-collected amphipods must be a static, 96-h LC50 using reagent-grade cadmium chloride. This test must be initiated within one day of the start of the 10-day test for sediment toxicity, and is normally started on the same day (EC, 1992).

The reference toxicity test requires a minimum of six treatments (i.e., a control and five concentrations of cadmium in water), and one or more replicates per treatment (USEPA, 1994a). The test is performed in 1-L glass beakers or jars, using $\geq$800 mL of test solution and a minimum of 10 amphipods per test chamber. Unless otherwise described, all applicable conditions and procedures for preparing for and undertaking the test must be identical to those defined in Sections 2, 3, and 4 of this report, except that sediment is not added to the test chambers and replicates are not required for each test concentration. One distinction is that, unlike the sediment toxicity test which requires continuous overhead illumination of test chambers, the reference toxicity test is to be performed in the dark (USEPA, 1994a). This can be achieved by covering test chambers with opaque material (e.g., aluminum foil), or by undertaking the test in a separate, enclosed testing facility where the lights are left off. A second distinction is that, unlike the sediment toxicity test, which requires gentle aeration of the overlying water throughout the test, the solutions of cadmium or water (control) in the test chambers are not aerated since the concentrations of dissolved oxygen that are present in each test solution (including the controls) are adequate to satisfy the oxygen requirements of the test organisms. Each test chamber is covered to minimize contamination and losses due to evaporation.

When undertaking a reference toxicity test, a series of concentrations should be chosen which, based on preliminary and/or previous tests performed using the same conditions and procedures, will provide partial mortalities in two or more concentrations and enable calculation of a 96-h LC50 with acceptably narrow 95% confidence limits. The selected test concentrations should bracket the predicted LC50 for the test species (see Appendices D, E, F, and G for species-specific guidance). An appropriate dilution series in which each successive concentration of cadmium is at least 50% of the previous concentration may be used.

Test concentrations may also be selected from other appropriate logarithmic dilution series (see Environment Canada, 1992; Appendix L).

The same type (i.e., natural or reconstituted seawater), source, and pretreatment of the control/dilution water should be used for each reference toxicity test performed by the laboratory using this procedure and a single species of test organisms. Salinity of this water must be 28 ± 2‰, and should be the same for each reference toxicity test performed with a particular species at each test facility. The control/dilution water must be temperature adjusted (i.e., 10 ± 2°C if *A. virginiana*; 15 ± 2°C if *R. abronius, E. washingtonianus,* or *E. estuarius*) and aerated as required to achieve a dissolved oxygen content of 90 to 100% saturation, before test solutions are made up and before each group of animals is introduced. The temperature of the solution in each test chamber should be measured daily, and must be measured at the beginning and end of the test. Mean daily temperature during the test must be 15 ± 2°C for *R. abronius, E. washingtonianus,* or *E. estuarius*; and 10 ± 2°C for *A. virginiana*. Dissolved oxygen, salinity, and pH in each test chamber must be measured at the beginning and end of the test.

At the end of the 96-h exposure period, the number of amphipods alive and the number dead are determined (see Section 4.9) and recorded for each treatment including the control group. Biological endpoints for this test are percent survival for each treatment, and the 96-h LC50. Environment Canada (1998a) provides definitive direction and advice for calculating LC50s, which should be followed. Results must be calculated and reported as *mg Cd/L*.

For tests using *R. abronius*, *E. estuarius*, or *A. virginiana*, the results of the reference toxicity test are only valid and acceptable if control survival at 96 h is ≥90% (EC, 1992; USEPA, 1994a). For tests using *E. washingtonianus*, the results of the reference toxicity test are only valid and acceptable if control survival at 96 h is ≥85% (see Appendix E).

It is the responsibility of laboratory personnel to demonstrate their ability to obtain consistent, precise results with the reference toxicant before definitive sediment assays are conducted using this reference method. To meet this responsibility, the laboratory personnel should initially determine their intralaboratory precision, expressed as percent coefficient of variation (% CV), by performing five or more reference toxicity tests with different batches of test organisms of the same species, using cadmium chloride and the procedures and conditions defined herein. This should be conducted to gain experience with the test procedure, and as a point of reference for future tests (USEPA, 1994a).

While routinely performing this reference toxicity test with each batch of field-collected amphipods of the same species, laboratory personnel should continue to follow this same procedure. Once sufficient data are available (EC, 1990), LC50s derived from these tests must be plotted successively on a species-specific warning chart, and examined to determine whether the results are within ± 2 SD of values obtained in previous tests using the same species, reference toxicant (i.e., cadmium chloride), and test procedure. A separate warning chart must be prepared and updated for each species of marine or estuarine amphipod used with this reference method.

The warning chart should plot 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 must be compared with established limits of the chart; the LC50 is acceptable if it falls within the warning limits. All calculations of mean and standard deviation should be made on the basis of log(LC50).

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 until the statistics stabilize (EC, 1990; 1998a; USEPA, 1994a).

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 mean abnormal sensitivity of the batch of test organisms or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all acclimation and test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, or to obtain a new batch of field-collected organisms for evaluating the toxicity of the samples of test material (together with a new reference toxicity test using the new batch of test organisms).

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).

Section 6

Data Analysis and Interpretation

6.1 Data Analysis

Investigators should consult Environment Canada (1998a) as well as Section 12 in USEPA (1994a) for detailed guidance regarding the appropriate statistical endpoints and their calculation.

The objective of the data analysis is to quantify contaminant effects on replicate (see Section 4.1) groups of test organisms exposed to various treatments of concern, and to determine if these effects are statistically different from those occurring in a reference or control sediment. Initially, statistical endpoints (i.e., mean ± SD, for percent survival at Day 10; see Section 4.10) are calculated for the replicate samples representing each treatment (including those representing the reference and control treatments). Each study consists of at least a control treatment (i.e., five or more groups of amphipods exposed to control sediment from the site where test organisms were collected) and one or more test treatments. A test treatment might be represented by replicate samples of dredged material from a particular depth or locale (sampling station) of interest, or replicate samples of field-collected sediment from a particular station within or adjacent to an ocean disposal site. Alternatively, a test treatment might be represented by five or more subsamples (i.e., laboratory replicates) of a single (nonreplicated) sample of sediment from a particular sampling station or site-specific depth (see Section 4.1). In each case, each test treatment is normally represented by ≥ 5 replicates. The same number of replicates per treatment should be used in the test wherever possible, to maximize statistical power and robustness.

Each study with samples of test sediment should, if possible, include one or more reference stations, for which ≥ 5 replicate samples or subsamples would be included in the toxicity test (Section 4.1). Statistical comparisons of biological data for the replicates representing each test treatment (i.e., potentially contaminated sediment from a single sampling station and depth) with that for replicate samples of reference sediment, should be applied whenever possible or appropriate (EC, 1992; EC, 1998a; 1998b). Such comparisons provide a site-specific basis for evaluating toxicity. Statistical comparisons of biological data for test sediment(s) with that for the control sediment(s) should be made if the samples of reference sediment prove unsuitable for comparison with samples from other sites (e.g., due to their toxicity or physicochemical characteristics that are atypical of test sediments), or if the control sediment(s) used are more appropriate for distinguishing contaminant effects from effects due to confounding factors such as sediment grain size. Regardless of whether or not statistical comparisons are made with reference sediment or control sediment, the experimental results obtained using control sediment must be used as a criterion for judging the validity and acceptability of the test (Section 4.6).

Samples of reference sediment must first meet the species-specific application limits for porewater salinity and grain size (see Section 2.6), if they are to be incorporated in

a sediment toxicity test. Given the species-specific criteria for test validity included herein (Section 4.6), which reflect a somewhat differing ability of the four candidate test species to survive a 10-day exposure to uncontaminated sediment, the following recommendations are made for judging whether or not to compare the endpoint results for the test sediments against those for the reference sediment:

- For *R. abronius* or *E. estuarius,* the mean 10-day survival rate in the replicate samples of reference sediment must be at least 80% to be eligible and acceptable for comparison with results for test sediments.

- For *E. washingtonianus*, the mean 10-day survival rate in the replicate samples of reference sediment must be at least 75% to be eligible and acceptable for comparison with results for test sediments.

- For *A. virginiana*, the mean 10-day survival rate in the replicate samples of reference sediment must be at least 70% to be eligible and acceptable for comparison with results for test sediments.

The recommendations for judging the suitability of comparisons of toxicity data for test sediment(s) with reference sediment use a species-specific mean survival rate 10% lower than the respective species-specific criterion for a valid test which is based on the mean 10-day survival rate in control sediment (see Section 4.6). In each instance, this permits a similar and somewhat (i.e., up to 10%) lower minimum mean 10-day survival rate in reference sediment relative to that in control sediment. This allowance is provided in consideration of the possibility of some reduced survival in reference sediment due to its dissimilar physicochemical characteristics (e.g., grain size), compared to sediment that the test organisms are accustomed to (i.e., control sediment).

The statistical procedures and interpretation of the results should be appropriate to the experimental design and study intent (see USEPA/USACE, 1991; USEPA, 1994a; EC, 1998a; and EC, 1998b for further guidance). Using this reference method, pairwise comparisons of survival data for each test treatment are normally made against survival data derived for a particular reference or control sediment. Initially, all data should be tested for normality using the *Shapiro-Wilk's test*, and for homogeneity of variance using *Bartlett's test* or other suitable tests (USEPA, 1994a). These and other statistical procedures are included in the methods of "TOXSTAT"; a series of statistical programs on computer disk which can be purchased by contacting WEST, Inc. (2003 Central Avenue, Cheyenne, WY, USA). Instructions for use accompany the TOXSTAT programs on disk.

Survival data which pass the tests for normality and homogeneity of variance should be treated by a pairwise comparison of the results for each test treatment versus the results for the reference or control treatment (see earlier discussion). A one-tailed *Student's t-test* should be used for this purpose. If a set of data cannot meet the requirements for normality and homogeneity of variance, an arcsine-square root transformation should be applied, followed by retesting for both (USEPA, 1994a). If the transformed data do not meet the assumption

of normality, nonparametric statistics such as the *Wilcoxon Rank Sum Test* (USEPA, 1994a) or other suitable tests can be applied. If the transformed data meet the assumption of normality, Bartlett's test or *Hartley's F test* should be used to test the homogeneity of variance assumption. Failure of the homogeneity of variance assumption leads to the use of a modified one-tailed Student's *t*-test, with adjusted degrees of freedom (USEPA, 1994a). Transformed data which meet the requirements for both normality and homogeneity of variance should be treated by a straightforward pairwise comparison using a one-tailed Student's *t*-test.

6.2 Interpretation of Results

Interpretation of results is not necessarily the sole responsibility of the laboratory personnel undertaking the test; this might be a shared task which includes an environmental consultant or other qualified persons responsible for reviewing and interpreting the findings.

Environment Canada (1998b) provides useful advice for interpreting and applying the results of toxicity tests with environmental samples; and should be referred to for guidance in these respects. Initially, the investigator should examine the results and determine if they are valid. In this regard, the species-specific criteria for a valid test (see Section 4.6) must be met.

The findings of the reference toxicity test which was initiated with the same batch of organisms as those used in the sediment toxicity test (see Section 5) should be considered during the interpretive phase of the investigation. These results, when compared with historic test results derived by the testing facility using the same reference toxicant, test organism, and test procedure (i.e., by comparison against the laboratory's warning chart for this reference toxicity test), will provide insight into the sensitivity of the test organisms as well as the laboratory's testing precision and performance at the time that the sediment toxicity test was conducted.

All data representing the known physicochemical characteristics of each sample of test material (including that for control and reference sediment) should be reviewed and considered when interpreting the results. The analytical data determined for whole sediment and pore water (see Section 4.3) should be compared with the known tolerance limits and application limits for the species of amphipod used in the test (see Appendices D for *R. abronius*, E for *E. washingtonianus*, F for *E. estuarius*, and G for *A. virginiana*). Values which approach (but do not exceed) the known tolerance limits (e.g., for ammonia) or application limits (i.e., for sediment grain size or porewater salinity) for each species could reduce their tolerance to contaminants within the sample, and thus have influenced the test results.

Concentrations of porewater ammonia and/or hydrogen sulphide can be elevated in samples of dredged material or field-collected estuarine or marine sediment. The elevated levels might be due to organic enrichment from natural and/or anthropogenic (man-made) sources. The known tolerance limits of the species of test organism used in the study should be considered together with the measured levels of these toxic constituents, when appraising their significance in influencing the test

results. Measured concentrations of ammonia should be converted to those for un-ionized ammonia (based on test conditions of pH and temperature), and the concentrations for both total ammonia and un-ionized ammonia considered with respect to reported tolerance limits for this chemical.

All physicochemical data determined for the overlying water during the sediment toxicity test (see Section 4.8) should also be reviewed and considered when interpreting the findings. If, for example, records indicate that the dissolved oxygen concentration in the overlying water within one or more test chambers fell to levels below 60% of saturation, this oxygen depression might have contributed to any toxic responses observed therein (ASTM, 1993; USEPA, 1994a). Similarly, any recorded excursions in water temperature beyond the allowable limits (Section 4.5) should be identified and assessed in conjunction with the test results and their interpretation. As with the porewater ammonia analyses, measurements of ammonia in overlying water at the start and end of the test (Section 4.8) should also be converted to the respective values for un-ionized ammonia (based on the concurrent measurements of pH and temperature for the overlying water). These values should be considered together with the known species' tolerance to ammonia, when interpreting the test results.

Records of numbers of animals seen swimming in the overlying water, floating on its surface, or emerged from the sediment during the test (Section 4.8) should be reviewed and considered together with those indicating any initial avoidance responses during the first hour of the test (Section 4.7). Any evidence of an avoidance response to one or more samples or treatments should be appraised and interpreted in conjunction with the physicochemical results (whole sediment and pore water; Section 4.3) for the same samples.

The purpose of this sediment toxicity test is to determine whether one or more test sediments are toxic to the test organisms, using the conditions and procedures herein. Various criteria have been used by researchers and regulators to judge if samples of test sediment pass or fail a 10-day toxicity test using marine or estuarine amphipods. For instance, some investigators have ranked test sediment as toxic if 10-day survival was significantly different from that of controls, based on the results of a Student's *t* test (Schlekat *et al.*, 1995). Others have concluded that mean 10-day survival rates in test sediment which are less than 80% (using *R. abronius*) and statistically different from that in reference sediment should be considered significant (Scott *et al.*, 1990). USEPA (1994b) states that "*...dredged material does not meet the benthic toxicity criteria if mortality rates for the dredged material tests exceed that of the reference sediment by more than 20% for amphipods (20% represents the minimum detectable difference of the test method)*". Interim guidelines by Environment Canada researchers and regulators have largely followed USEPA (1994b) when judging if test sediments pass or fail a 10-day test for sediment toxicity using marine or estuarine amphipods, but have also incorporated an alternate criterion based on comparison of results with control sediment in the absence of an acceptable reference sediment (Lee *et al.*, 1995; EC, 1997).

In keeping with Environment Canada (1997), the following two-part guidance is

recommended when judging if samples of test sediment pass or fail a 10-day test for sediment toxicity, using this reference method:

- Test sediment from a particular sampling station and depth is judged to have failed this sediment toxicity test if the mean 10-day survival rate for the replicate groups of test organisms exposed to this sediment is more than 20% lower than that in the reference sediment and is significantly different.

- In the absence of an acceptable reference sediment, the test sediment is judged to have failed this sediment toxicity test if the mean 10-day survival rate for the replicate groups of test organisms exposed to this sediment is more than 30% lower than that in the control sediment and is significantly different.

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 reference method, and, if so, provide details of the 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 the items which 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 or regulations might require selected test-specific items listed in Section 7.2 (e.g., details regarding 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 in this report, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

Details on the conduct and findings of the test, which are not conveyed by the test-specific report or general report, should 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) which are 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 on their collection, transport, holding, acclimation, and health; and
- information on the calibration of equipment and instruments.

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

7.1 Minimum Requirements for a Test-specific Report

Following is a list of items that must be included in each test-specific report.

7.1.1 Test Material

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

7.1.2 Test Organisms

- species, source, and date of collection; 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 start of the toxicity test.

7.1.5 Test Method

- citation of biological test method used (i.e., as per this report);
- frequency and type of measurements and 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 report;
- 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);
- volume of sediment and overlying water in each test chamber;
- number of organisms per test chamber and treatment;
- dates when test was started and ended;
- for each sample — percent very coarse-grained sediment (i.e., particles >1.0 mm), percent sand, percent silt, percent clay, percent water content, total organic carbon; porewater salinity, porewater pH, and porewater ammonia;
- for at least one test chamber representing each treatment — all measurements of temperature, dissolved oxygen, salinity, ammonia, and pH in overlying water; and
- any measurements showing DO <60% saturation in the overlying water, for any test chamber.

7.1.7 Test Results

- mean (± SD) percentage of amphipods that survived in each treatment (including the control) during the 10-day test, together with the results of all pairwise statistical comparisons;

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

- anything unusual about the test, any deviation from these procedures and conditions, any problems encountered, any remedial measures taken.

7.2 Additional Reporting Requirements

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

7.2.1 Test 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

- collection site and supplier of organisms;

- conditions and procedures during collection (e.g., tide status, sea conditions, collection by boat or beach seine, collection by dredge or shovel, sieving and handling procedures in the field);

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

- estimated number of amphipods transferred to each collection/holding/acclimation container, as provided by collector(s); any observations of condition, appearance, and behaviour of amphipods when received at testing laboratory; numbers of dead, atypical, or apparently unhealthy animals removed from each collection/holding/acclimation container during the period preceding sieving and collection of animals to be placed in test chambers;

- description of holding and acclimation conditions (facilities; lighting source and intensity at surface of overlying water in holding/acclimation containers; seawater source and quality; water pretreatment; water exchange rate and estimated density of amphipods in holding/acclimation containers; salinity, temperature, dissolved oxygen, and pH during holding and acclimation);

- average total body length (with range and sample size) of individual amphipods used in the test; and

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

7.2.3 Test Facilities and Apparatus

- description of laboratory's previous experience with this reference method using the selected species of test organism;
- description of systems for providing lighting and compressed air, and for regulating temperature within test facility;
- description of test chambers; and
- description of procedures used to clean and rinse test apparatus.

7.2.4 Test Water

- type and quantity of any chemical(s) added to test water;
- procedure for adjusting salinity, temperature, and dissolved oxygen; and
- storage conditions and duration before use.

7.2.5 Test Method

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

7.2.6 Test Conditions and Procedures

- measurements of light intensity adjacent to surface of overlying water in test chambers;
- statement concerning conditions (rate and manner) for aerating overlying water in test chambers before and during the test;
- records of any disruption of air flow to test chambers, and of related DO measurements;
- appearance of each sample 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 control or reference sediment); e.g., faunal tracks, qualitative and/or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses; and
- chemical analyses of concentrations of cadmium in test solutions of reference toxicant.

7.2.7 Test Results

- records of observations of amphipod appearance and behaviour when initiating the test and during its first hour (i.e., apparent avoidance responses); records of numbers of amphipods replaced during this period;

- records of numbers of animals seen swimming in the water, floating on its surface, moving on the surface of the sediment, or emerged from the sediment but apparently dead, during each observation period; records of numbers surviving, dead, and missing (and presumed dead) at test end;

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

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Appendix D

Rhepoxynius abronius — Known Tolerance and Application Limits

Tolerance Limits for Reference Toxicant

Since 1988, Environment Canada's Atlantic and Pacific regional laboratories have been undertaking "water-only" 96-h LC50 reference toxicity tests with each group of field-collected *Rhepoxynius abronius* used in 10-day sediment toxicity tests. Results for these tests (n = 15), performed according to Environment Canada (1992) and Section 5 herein, have been plotted and summarized as warning limits (geometric mean ± 2 SD; Doe, 1997; Fennell, 1997). These summary values (Table D.1), should help guide inexperienced laboratories in selecting an appropriate range of test concentrations for undertaking reference toxicity tests with this species; and are useful for comparative purposes. Similar tolerances of *R. abronius* to cadmium, in 96-h "water-only" LC50s, have been reported elsewhere (e.g., DeWitt *et al.*, 1989).

Tolerance and Application Limits for Salinity

R. abronius is very intolerant of low salinity. Swartz *et al.* (1985) reported that the 10-day survival of this species was reduced significantly when porewater salinity was 18.8‰ , and that no amphipods survived at salinities of 9.9‰ and 12.3‰. In a separate series of studies, Swartz *et al.* (1985) determined that the mean survival of *R. abronius* at interstitial salinity of 15‰ was significantly less than at 25‰. Based on their studies of salinity tolerance, these authors concluded "*Conservatively, interstitial salinity of test sediment should be at least 25‰ before salinity effects on survival can be discounted"*. They also concluded (Swartz *et al.*, 1985) "*The sensitivity of* R. abronius *to salinity effectively limits the application of this test procedure to field sediment samples collected from the coastal zone and higher salinity portions of estuaries. Attempts to raise interstitial salinity by adding or sieving into higher salinity water are likely to change the toxicological properties of the sample*."

Lee and Fennell (1995) re-examined the salinity tolerance of *R. abronius*. Ten-day survival tests were performed using Target™ "superfine" (≤0.5 mm) sand and seawater with overlying salinity adjusted to values of 15‰, 20‰, 25‰, 30‰, 35‰, 40‰, and 45‰. Mean survival was 70 to 83% at salinities ranging from 25 to 35‰; these values did not differ significantly. Survival at 15‰ was 0%, and at 20‰ was only 31%. Mean survival rates at 40‰ and 45‰ were 32% and 39%, respectively.

USEPA (1994a) states that a "salinity tolerance range" of 25 to 32‰ is indicated for this species. For 10-day tests, USEPA (1994a) has specified an "application limit" of >25‰ for the overlying water.

Table D.1 Known Tolerance and Application Limits for Ten-day Tests for Sediment Toxicity Using *Rhepoxynius abronius*

Parameter	Known Tolerance Limits	Application Limits
96-h "water-only" LC50 for reference toxicant (mg Cd/L)	0.6 (0.2 to 1.9) [a] 0.6 (0.4 to 1.1) [b]	
porewater salinity (‰)	25 to 35	must be 25 to 35
% very coarse-grained sediment [c]		0 to 100 is acceptable
% fines [d]		must be <90
% clay [e]		must be <40
96-h "water-only" LC50, total ammonia (mg N/L)	65.0 (40.4 to 89.5) [f]	
96-h "water-only" LC50, un-ionized ammonia (mg N/L)	1.1 (0.7 to 1.4) [f]	
10-day porewater LC50, total ammonia (mg N/L)	57.7 (51.9 to 63.6) [f]	
10-day porewater LC50, un-ionized ammonia (mg N/L)	1.4 (1.3 to 1.5) [f]	
porewater hydrogen sulphide (mg/L)	not available	

[a] Geometric mean (± 2 SD) for 15 tests performed at Environment Canada's Atlantic regional laboratory.
[b] Geometric mean (± 2 SD) for 15 tests performed at Environment Canada's Pacific regional laboratory.
[c] Percentage of particles in test material >1.0 mm.
[d] Percentage of particles in test material <0.063 mm (i.e., % silt and clay).
[e] Percentage of particles in test material <0.004 mm.
[f] LC50 with 95% confidence limits in parentheses; based on measured (total ammonia-nitrogen) and calculated-from- measured (un-ionized ammonia-nitrogen; Bower and Bidwell, 1978) concentrations. From Tay *et al.* (1998).

Based on the findings of Swartz *et al.* (1985) and Lee and Fennell (1995), it is evident that *R. abronius* is tolerant of salinities ranging within 25‰ to 35‰. An *R. abronius* application limit of 25‰ to 35‰ is specified here for porewater salinity (see Table D.1 and Section 2.6). Test material with porewater salinity less than 25‰ must not be used for a 10-day sediment toxicity test with this species. Rather, such material should be evaluated for toxicity using another suitable species which is tolerant of low-salinity water (e.g., *Eohaustorius estuarius;* see Appendix F).

Tolerance Limits for High Organic Content

R. abronius is tolerant of substantial enrichment of sediment. Ten-day tests performed by Swartz *et al.* (1985) have shown that mean survival rates in samples of field-collected "clean" sediment with a total volatile solids content as high as 18% can be equivalent to that for control groups. Paine and McPherson (1991a) reported that 10-day survival rates of 89 to 92% were achieved when this species was held in a sample of field-collected sediment with a total organic carbon content of 10%, and that similar survival rates were found for uncontaminated sediment with a total organic content of 4%. Results for 10-day tests with field-collected sediment from 12 inlets off the west coast of the British Columbia mainland showed that sample survival rates (ranging from 35 to 88%) and total organic carbon content (ranging from 0.4 to 4.8%) were poorly correlated (R = 0.10) (Sullivan *et al.*, 1998a).

No studies with formulated sediments are available which show the effect of elevated levels of organic carbon on the 10-day survival rate for this species. Studies with commercial formulations of silica sand (Tay *et al.*, 1998) demonstrate that *R. abronius* can survive well for 10 days in the absence of any appreciable organic carbon content.

It is concluded that this species can tolerate samples of test material with a total organic carbon content of 18% or less. However, the upper limit that can be tolerated without affecting 10-day survival is not known. No application limit for total organic carbon seems necessary or appropriate.

Tolerance and Application Limits for Grain Size

The influence of sediment grain size on the 10-day survival rates for *R. abronius* in sediment toxicity tests has been considered in numerous studies. Most attention has focused on the influence of sediment fines (i.e., particles <0.063 mm), although the tolerance of this species to coarse-grained sediment has also been investigated (Lee, 1994; Tay *et al.*, 1998).

Some studies have demonstrated high 10-day survival rates for *R. abronius* exposed to field-collected reference sediments with a high percentage of fines and a high percentage of clay. For instance, Swartz *et al.* (1985) reported a mean 10-day survival rate of 90% for *R. abronius* held in a sample of sediment comprised of 10% sand, 37% silt, and 53% clay (90% fines). Similarly, Long *et al.* (1990) found a mean 10-day survival rate of 91% for *R. abronius* held in a sample of field-collected sediment with 48.3% silt and 48.4% clay (i.e., 96.7% fines); and McLeay *et al.* (1991) noted 10-day survival rates of 81 to 91% for amphipods held in a sample of reference sediment with 99% fines. Conversely, McLeay *et al.* (1991) found reduced survival rates in another sample of reference sediment from a separate site with 99% fines. Sullivan *et al.*

(1998b) reported a high (92%) 10-day survival rate for *R. abronius* held in a sample of field-collected sediment with 35% clay and 84% fines. Pinza *et al.* (1997) noted a mean 10-day survival rate of >90% when *R. abronius* were held in a sample of field-collected sediment comprised of 36% clay and 90% fines.

A number of studies have shown some reduction in 10-day survival rates when this species is held in fine-grained (predominantly silt and clay) field-collected reference sediment, or in commercial formulations of clay or silica sand:clay mixtures with a high percentage of fines. In a series of tests with various sand or clay formulations having markedly different grain size characteristics, Lee (1994) found that survival of *R. abronius* was highly negatively correlated (R = -0.82) with percent clay. Tay *et al.* (1998) demonstrated that, for this species, mean 10-day survival rates were reduced from a control value of 98% to ≤71% when sand:clay mixtures contained ≥22% clay. Similarly, Tay *et al.* (1998) found that a sample of reference sediment with 17% clay and 82% fines reduced survival to 75%. In 10-day tests with 12 samples of west coast sediments distant from and apparently unaffected by anthropogenic activities, Sullivan *et al.* (1998a) found that mean survival rates were <60% for four of seven samples with ≥90% fines; additionally, mean survival rates were <60% for five of eight samples with ≥40% clay.

Some (slight) reduction in tolerance of this species to very coarse-grained sand is evident from two series of studies with various sand:clay mixtures. Lee (1994) observed a slight reduction in mean 10-day survival from 90% (controls) to 84% for groups held in a commercial formulation ("silica sand no. 2") comprised of ~92% very coarse-grained (>1.0 mm) sediment. Tay *et al.* (1998) reported a similar finding for this mixture, with a mean survival rate of 77%.

In consideration of the evidence of some intolerance of *R. abronius* to a high percentage of fines, USEPA (1994a) has designated an application limit of <90% fines for this species. This application limit seems reasonable, and is adopted herein (see Table D.1 and Section 2.6). Additionally, a second application limit of <40% clay seems reasonable, and is to be applied as part of this reference method. Accordingly, test material with ≥90% fines and/or ≥40% clay content must not be used for a 10-day sediment toxicity test with *R. abronius* according to this reference method. Rather, another test species more tolerant of fine-grained sediment (e.g., *Eohaustorius estuarius*; see Appendix F) should be used, provided the application limits for this species are not exceeded. No application limit for very coarse-grained (i.e., >1.0 mm) material is necessary or appropriate for this species (Section 2.6).

Tolerance Limits for Ammonia

Sims and Moore (1995a) undertook a literature review for concentrations of ammonia in sediment pore water, as well as for known toxicity of ammonia to marine and freshwater invertebrates and fish. The authors concluded "*The comparison of reported exposure and effects concentrations suggests significant potential for ammonia toxicity in dredged material bioassays".*

Tay *et al.* (1998) measured the tolerance of *R. abronius* to ammonia in both a 96-h "water- only" test and a 10-day "spiked sediment" test. In each instance, LC50s were calculated and expressed based on both measured total ammonia and calculated (Bower and Bidwell, 1978) un-ionized

ammonia concentrations. Values derived for these tests are shown in Table D.1. Results show similar respective values (i.e., as total ammonia or un-ionized ammonia) for the 96-h "water-only ammonia" and 10-day "spiked sediment/porewater ammonia" tests.

Tay *et al.* (1998) found a 96-h "water-only" LC50 for total ammonia of 65.0 mg N/L. This value is identical to the water-only 96-h LC50 for total ammonia of 65 mg N/L reported by Kohn *et al.* (1994), and similar to the mean 96-h LC50 (n = 6) for total ammonia of 58 mg N/L reported by Pinza *et al.* (1997). The 96-h LC50 of 1.1 mg N/L for un-ionized ammonia calculated by Tay *et al.* (1998) is also similar to the value for un-ionized ammonia of 1.3 mg N/L given in Kohn *et al.* (1994). Other than those in Tay *et al.* (1998) (see Table D.1), no reports of 10-day LC50s for ammonia (total or un-ionized) were found in the literature reviewed for this species.

USEPA (1994a) presents *R. abronius* application limits for both total ammonia and un-ionized ammonia in sediment. These values, identified as "water column no-effect concentrations", are <30 mg NH_3/L (= <24.7 mg N/L) as total ammonia, and <0.4 mg NH_3/L (= <0.3 mg N/L) as un-ionized ammonia (pH 7.7).

No application limits are imposed here for total or un-ionized ammonia in test materials (see Table D.1), inasmuch as the ammonia concentrations in samples under investigation might be elevated due to anthropogenic and/or natural causes, and might be an integral toxic component deserving of consideration using this species and test method.

Tolerance Limits for Hydrogen Sulphide

Hydrogen sulphide can be elevated in sediment pore water to levels toxic to amphipods and other benthic life (Sims and Moore, 1995b). Based on a literature review of measured porewater concentrations of hydrogen sulphide and its known toxicity to marine or freshwater organisms, these authors concluded "*The comparison of reported exposure and effects concentrations suggests a strong potential for hydrogen sulphide toxicity in dredged material bioassays"*. To date, however, no definitive data are available showing the limits of hydrogen sulphide in pore water or overlying water (i.e., results of "water-only LC50s) that *R. abronius* can tolerate.

Historical Control Performance

For more than a decade, many North American laboratories have undertaken numerous 10-day sediment toxicity tests and 96-h *water only* reference toxicity tests with this species. Mean 10-day survival rates in control sediment have routinely been ≥90% in the majority of instances. Additionally, the *water only* controls for reference toxicity tests have typically achieved ≥90% survival during 96-h exposures. Accordingly, minimum mean 10-day survival rates of ≥90% in control sediment, and 96-h survival rates of ≥90% in the control/dilution water used in reference toxicity tests, are considered to be readily achievable and suitable limits on which to base criteria for valid sediment and reference toxicity tests using *R. abronius* (see Sections 4.6 and 5).

Appendix E

Eohaustorius washingtonianus — Known Tolerance and Application Limits

Tolerance Limits for Reference Toxicant

Environment Canada's Pacific regional laboratory has been undertaking "water-only" 96-h LC50 reference toxicity tests with each group of field-collected *Eohaustorius washingtonianus* used in 10-day sediment toxicity tests. Results for 29 tests performed from November 1994 to April 1997 have been plotted and summarized as warning limits (geometric mean ± 2 SD; Fennell, 1997) according to Environment Canada (1992) and Section 5 herein. These summary values (Table E.1) should help guide inexperienced laboratories in selecting an appropriate range of test concentrations for undertaking reference toxicity tests with this species; and are useful for comparative purposes. Similar tolerances of *E. washingtonianus* to cadmium, in 96-h "water-only" LC50s performed at other laboratories, have been reported elsewhere (Paine and McPherson, 1991b).

Tolerance and Application Limits for Salinity

Lee and Fennell (1995) reported the findings of a series of ten-day survival tests which were undertaken to determine the tolerance of *E. washingtonianus* to a range of concentrations of porewater salinity. Each assay was performed using dry Target™ "superfine" (≤0.5 mm) sand and seawater with overlying salinity adjusted to values of 15‰, 20‰, 25‰, 30‰, 35‰, 40‰, and 45‰. Mean 10-day survival was not significantly different throughout the range 15 to 35‰; mean values within this range were 83 to 94% and no salinity-dependent trend was apparent. At salinities of 40‰ and 45‰, mean survival rates were significantly lower (i.e., 60% and 43%, respectively). No other studies are available which report the salinity tolerance of this species.

Based on the findings of Lee and Fennell (1995), it is evident that *E. washingtonianus* is tolerant of salinities ranging within 15 to 35‰. An *E. washingtonianus* application limit of 15 to 35‰ is specified here for porewater salinity (see Table E.1 and Section 2.6). Test material with porewater salinity less than 15‰ must be evaluated for toxicity using another suitable species which is more tolerant of low-salinity water (e.g., *Eohaustorius estuarius;* see Appendix F).

Tolerance Limits for High Organic Content

No studies with formulated sediments are available which demonstrate the effect of high organic carbon content on the 10-day survival rate for this species. Results for tests with uncontaminated reference sediments are also not enlightening in this respect, inasmuch as no reports are available which show 10-day survival rates for *E. washingtonianus* when exposed to samples with organic content greater than 5%. Studies with commercial formulations of silica sand (Tay *et al.*, 1998) demonstrate that *E. washingtonianus* can survive well for 10 days in the absence of any appreciable organic carbon content.

Table E.1 Known Tolerance and Application Limits for Ten-day Tests for Sediment Toxicity Using *Eohaustorius washingtonianus*

Parameter	Known Tolerance Limits	Application Limits
96-h "water-only" LC50 for reference toxicant (mg Cd/L)	0.5 (0.4 to 0.8) [a]	
porewater salinity (‰)	15 to 35	must be 15 to 35
% very coarse-grained sediment [b]		must be <25
% fines [c]		must be <80
% clay [d]		must be <20
96-h "water-only" LC50, total ammonia (mg N/L)	139 (111 to 167) [e]	
96-h "water-only" LC50, un-ionized ammonia (mg N/L)	1.9 (1.7 to 2.2) [e]	
10-day porewater LC50, total ammonia (mg N/L)	112 (86.3 to 138) [e]	
10-day porewater LC50, un-ionized ammonia (mg N/L)	1.6 (1.3 to 1.8) [e]	
porewater hydrogen sulphide (mg/L)	not available	

[a] Geometric mean (± 2 SD) for 29 tests performed at Environment Canada's Pacific regional laboratory.
[b] Percentage of particles in test material >1.0 mm.
[c] Percentage of particles in test material <0.063 mm (i.e., % silt and clay).
[d] Percentage of particles in test material <0.004 mm.
[e] LC50 with 95% confidence limits in parentheses; based on measured (total ammonia-nitrogen) and calculated-from- measured (un-ionized ammonia-nitrogen; Bower and Bidwell, 1978) concentrations. From Tay *et al.* (1998).

Results for 10-day tests with "clean" reference sediment from 12 inlets off the west coast of the British Columbia mainland showed that sample survival rates (ranging from means of 10 to 95%) and total organic carbon content (ranging from 0.4 to 4.8%) were poorly correlated (R = 0.23) (Sullivan *et al.*, 1998a). Sullivan *et al.* (1998a) found a high mean 10-day survival rate of 80% in a sample with 4.8% organic content. Similarly, Lee *et al.* (1995) noted *E. washingtonianus* survival rates as high as 80% in field-collected sediment with 4.5% organic content.

It appears that *E. washingtonianus* can tolerate samples of test material with a total organic carbon content of 5% or less, but the upper limit that can be tolerated without affecting 10-day survival is not known. No application limit for total organic carbon seems necessary or appropriate.

Tolerance and Application Limits for Grain Size

Two studies by Environment Canada researchers (Lee, 1994; Tay *et al.*, 1998) have examined the influence of sediment grain size on the 10-day survival of *E. washingtonianus*. Each of these studies was undertaken using various mixtures or commercial formulations of silica sand and clay. The findings of Lee (1994) demonstrated that mean survival rates were decreased to 9% or 55% when this species was exposed to silica sand formulations with ~92% or ~27%, respectively, of very coarse-grained (i.e., >1.0 mm) sediment. Tay *et al.* (1998) also demonstrated that this species was intolerant of a high percentage of very coarse-grained sediment, inasmuch as mean 10-day survival was reduced from a control value of 98% to 60% when amphipods were held in "silica sand no. 2", comprised of ~92% very coarse-grained (i.e., >1.0 mm) sediment. Based on these findings, an *E. washingtonianus* application limit of <25% very coarse-grained sediment is to be applied as part of this reference method (see Table E.1 and Section 2.6). Accordingly, test material with ≥25% very coarse-grained (i.e., >1.0 mm) sediment must not be used for a 10-day sediment toxicity test with *E. washingtonianus* according to this reference method. Rather, another test species more tolerant of a high percentage of very coarse-grained sediment (e.g., *R. abronius, E. estuarius,* or *A. virginiana*; see Appendices D, F, and G) should be used.

The studies with commercial formulations of sand, clay, or sand:clay mixtures by Lee (1994) and Tay *et al.* (1998) each demonstrate that *E. washingtonianus* is very intolerant of a high percentage of fine-grained (<0.063 mm) material. Lee (1994) found a mean 10-day survival rate of only 14% when this species was exposed to a formulation of 95% fines and 59% clay; and survival was 0% for a formulation with 99% fines and 84% clay. Tay *et al.* (1998) found that mean 10-day survival rates in sand:clay mixtures declined progressively with increasing clay content, from 43% survival for 22% clay (31% fines) to only 17% survival for 64% clay (99% fines).

A number of studies with "clean" field-collected reference sediments support the findings for commercial sand/clay formulations that this species is intolerant of a high percentage of fine-grained material. In 10-day tests with 12 samples of west coast sediments distant from and apparently unaffected by anthropogenic activities, Sullivan *et al.* (1998a) found that mean survival rates were ≤60% for seven of eight samples with ≥80% fines; additionally, mean survival rates were ≤60% for eight of ten samples with ≥30% clay. A reasonably-high negative

correlation (R = -0.76) between % clay and mean percent survival was found for these sediments. Lee *et al.* (1995) compared data for mean percent survival of *E. washingtonianus* in 34 samples of reference or contaminated field-collected sediment, and found that all samples with clay content ≥20% (20 of 34) had ≤80% survival. Similarly, these data showed that all samples with ≥55% fines (i.e., particles <0.063 mm) had ≤80% survival. For these (Lee *et al.*, 1995) data, survival rates were negatively correlated with clay content (R = -0.85).

Given the apparent intolerance of *E. washingtonianus* to a high percentage of fines, an *E. washingtonianus* application limit of <80% fines is designated here (see Table E.1 and Section 2.6). Additionally, a second application limit of <20% clay seems reasonable, and is to be applied as part of this reference method. Accordingly, test material with ≥80% fines and/or ≥20% clay content must not be used for a 10-day sediment toxicity test with *E. washingtonianus* according to this reference method. Rather, another test species more tolerant of fine-grained sediment (e.g., *Eohaustorius estuarius*; see Section 2.6) should be used, provided that grain size characteristics are within the application limits for this species.

Tolerance Limits for Ammonia

Sims and Moore (1995a) undertook a literature review for concentrations of ammonia in sediment pore water, as well as for known toxicity of ammonia to marine and freshwater invertebrates and fish. The authors concluded "*The comparison of reported exposure and effects concentrations suggests significant potential for ammonia toxicity in dredged material bioassays".*

Tay *et al.* (1998) measured the tolerance of *E. washingtonianus* to ammonia in both a 96-h "water- only" test and a 10-day "spiked sediment" test. In each instance, LC50s were calculated and expressed based on both measured total ammonia and calculated (Bower and Bidwell, 1978) un-ionized ammonia concentrations. Values derived for these tests are shown in Table E.1. Results show similar respective values (i.e., as total ammonia or un-ionized ammonia) for the 96-h "water-only ammonia" and 10-day "spiked sediment/porewater ammonia" tests. No other studies are available which show the acute lethal tolerance of this species to ammonia.

No application limits are imposed here for total or un-ionized ammonia in test materials (see Table E.1), inasmuch as the ammonia concentrations in samples under investigation might be elevated due to anthropogenic and/or natural causes, and might be an integral toxic component deserving of consideration using this species and test method.

Tolerance Limits for Hydrogen Sulphide

Hydrogen sulphide can be elevated in sediment pore water to levels toxic to amphipods and other benthic life (Sims and Moore, 1995b). Based on a literature review of measured porewater concentrations of hydrogen sulphide and its known toxicity to marine or freshwater organisms, these authors concluded "*The comparison of reported exposure and effects concentrations suggests a strong potential for hydrogen sulphide toxicity in dredged material bioassays".* To date, however, no definitive data are available which show the limits of hydrogen sulphide in pore water or overlying water (i.e., results of "water-only LC50s) which *E. washingtonianus* can tolerate.

Historical Control Performance

Environment Canada's Pacific regional laboratory has undertaken 37 separate series of 10-day sediment toxicity tests and associated 96-h *water only* reference toxicity tests with *E. washingtonianus* since late 1994. Mean 10-day survival rates in control sediment averaged 94%, and were consistently ≥85% for each test; although 12.9% of the tests failed to achieve ≥90% control survival (Fennell, 1998). For the associated reference toxicity tests, all but 5.4% of the control groups achieved ≥85% survival; whereas 13.5% of these tests failed to attain ≥90% control survival (Fennell, 1998). Given this historical control performance, minimum mean 10-day survival rates of ≥85% in control sediment, and 96-h survival rates of ≥85% in the control/dilution water used in reference toxicity tests, are considered to be readily achievable and suitable limits on which to base criteria for valid sediment and reference toxicity tests using *E. washingtonianus* (see Sections 4.6 and 5).

Appendix F

Eohaustorius estuarius — Known Tolerance and Application Limits

Tolerance Limits for Reference Toxicant

Environment Canada's Atlantic regional laboratory has been undertaking "water-only" 96-h LC50 reference toxicity tests with each group of field-collected *Eohaustorius estuarius* used in 10-day sediment toxicity tests. Results for ten tests, performed according to Environment Canada (1992) from November 1992 to May 1997, have been plotted and summarized as warning limits (geometric mean ± 2 SD; Doe, 1997). These summary values (Table F.1) should help guide inexperienced laboratories in selecting an appropriate range of test concentrations for undertaking reference toxicity tests with this species; and are useful for comparative purposes.

Other reports of findings for water-only reference toxicity tests using cadmium and *E. estuarius* are available in the literature. DeWitt *et al.* (1989) calculated a 96-h LC50 of 7.4 mg Cd/L; and Swartz *et al.* (1994) presented a value of 16.9 mg Cd/L. An interlaboratory study of the performance of 10-day sediment toxicity tests using this and other species of estuarine or marine amphipods, which involved eight laboratories and included a water-only toxicity test with cadmium by each, found a mean 96-h LC50 of 8.4 mg Cd/L for *E. estuarius*, with values for differing laboratories ranging from 4.8 to 11.2 mg Cd/L. These reported values are in keeping with those determined by Environment Canada's Atlantic regional laboratory (see Table F.1).

Tolerance and Application Limits for Salinity

E. estuarius is a euryhaline species that is very tolerant of a wide range of salinities. DeWitt *et al.* (1989) found that mean survival rates were consistently >95% at all salinities tested, in a series of 10-day tests where groups of *E. estuarius* were exposed to control sediment with porewater salinity adjusted to values ranging from 2 to 28‰. A subsequent unpublished study cited in USEPA (1994a) has shown that this species can tolerate salinities up to and including 34‰.

USEPA (1994a) states that a "salinity tolerance range" of 2 to 34‰ is indicated for this species. For 10-day sediment toxicity tests, USEPA (1994a) has specified an "application limit" of 0 to 34‰ for the overlying water.

Based on the known salinity tolerance range for this species, an *E. estuarius* application limit of 2 to 35‰ is specified here for porewater salinity (see Table F.1 and Section 2.6).

Tolerance Limits for High Organic Content

No studies with formulated sediments are available which demonstrate the effect of elevated levels of organic carbon on the 10-day survival rate for this species. Studies with commercial formulations of silica sand (Tay *et al.*, 1998) demonstrate that *E. estuarius* can survive well for 10

Table F.1 Known Tolerance and Application Limits for Ten-day Tests for Sediment Toxicity Using *Eohaustorius estuarius*

Parameter	Known Tolerance Limits	Application Limits
96-h "water-only" LC50 for reference toxicant (mg Cd/L)	5.3 (2.0 to 14.3) [a]	
porewater salinity (‰)	2 to 34	must be 2 to 35
% very coarse-grained sediment [b]		must be <90
% fines [c]		0 to 100 is acceptable
% clay [d]		must be <70
96-h "water-only" LC50, total ammonia (mg N/L)	156 (97.0 to 215) [e]	
96-h "water-only" LC50, un-ionized ammonia (mg N/L)	2.2 (1.6 to 2.9) [e]	
10-day porewater LC50, total ammonia (mg N/L)	96.8 (88.1 to 106) [e]	
10-day porewater LC50, un-ionized ammonia (mg N/L)	1.3 (1.1 to 1.4) [e]	
porewater hydrogen sulphide (mg/L)	not available	

[a] Geometric mean (± 2 SD) for 10 tests performed at Environment Canada's Atlantic regional laboratory.
[b] Percentage of particles in test material >1.0 mm.
[c] Percentage of particles in test material which are <0.063 mm (i.e., % silt and clay).
[d] Percentage of particles in test material which are <0.004 mm.
[e] LC50 with 95% confidence limits in parentheses; based on measured (total ammonia-nitrogen) and calculated-from-measured (un-ionized ammonia-nitrogen; Bower and Bidwell, 1978) concentrations. From Tay *et al.* (1998).

days in the absence of any appreciable organic carbon content. One study with a sample of field-collected sediment high in total organic carbon content (12.4%) showed that *E. estuarius* could tolerate this degree of enrichment (mean 10-day survival, 84%; Paine and McPherson, 1991a).

It is known that *E. estuarius* can tolerate samples of test material with a total organic carbon content of 12% or less; however, the upper limit that can be tolerated without affecting 10-day survival, is not known. No application limit for total organic carbon seems necessary or appropriate.

Tolerance and Application Limits for Grain Size

E. estuarius is tolerant of sediments with a wide range of grain size characteristics. Ten-day tests using a range of commercial formulations of silica sand, clay, or sand:clay mixtures with diverse grain sizes demonstrated that this species can show high survival rates in both coarse-grained and fine-grained sediments (Tay *et al.*, 1998). For instance, the mean 10-day survival rate in a sample of 100% silica sand comprised of ~27% very coarse-grained material (i.e., particles >1.0 mm) was 87%. However, mean 10-day survival was somewhat lower (71%) when amphipods of this species were held in ~92% very coarse-grained (>1.0 mm) sediment. Based on these findings, an *E. estuarius* application limit of <90% very coarse-grained sediment is to be applied as part of this reference method (see Table F.1 and Section 2.6. Accordingly, test material with ≥90% very coarse-grained (i.e., >1.0 mm) sediment must not be used for a 10-day sediment toxicity test with *E. estuarius* according to this reference method. Rather, another test species more tolerant of a high percentage of very coarse-grained sediment (e.g., *R. abronius* or *A. virginiana*; see Appendices D and G) should be used.

The tolerance of *E. estuarius* to commercial formulations of fine-grained material was shown by Tay *et al.* (1998) to be high, with mean survival rates of ≥82% for silica:clay mixtures with up to 57% clay and 79% fines. Some reduction in tolerance was evident, however, for a mixture with 64% clay and 99% fines, in which the mean survival rate was 74% (Tay *et al.*, 1998).

Results for 10-day toxicity tests with *E. estuarius* exposed to 42 samples of uncontaminated field sediment showed a slight decline in survival rate with an increasing percentage of fines; although correlations of survival versus percent fines (R = -0.22) and survival versus percent clay (R = -0.25) were low (DeWitt *et al.*, 1989). These tests, which included a high percentage of samples with >90% fines, showed an overall mean 10-day survival rate of 94.4%. Based on this and similar studies, USEPA (1994a) indicated that 10-day sediment toxicity tests with *E. estuarius* could be applied to sediments with a full range of grain size characteristics (unlike for *R. abronius*, for which an application limit of <90% fines was specified).

The *E. estuarius* application limit of "full range" (i.e., 0 to 100%) for percent fines presented in USEPA (1994a) seems reasonable without conflicting data, and is adopted herein (see Table F.1 and Section 2.6). Thus test sediments comprised of ≤100% fines may be included in 10-day sediment toxicity tests with this species. Given the findings by Tay *et al.* (1998) which demonstrate some reduction in survival of this species when exposed to commercial sand:silt:clay mixtures with a mean of 60% clay, an *E. estuarius* application limit of <70% clay is to be applied as part of this reference method. Accordingly, test material with ≥70% clay

content must not be used in a 10-day sediment toxicity test with *E. estuarius* according to this reference method.

Tolerance Limits for Ammonia

Sims and Moore (1995a) undertook a literature review for concentrations of ammonia in sediment pore water, as well as for known toxicity of ammonia to marine and freshwater invertebrates and fish. The authors concluded "*The comparison of reported exposure and effects concentrations suggests significant potential for ammonia toxicity in dredged material bioassays".*

Tay *et al.* (1998) measured the tolerance of *E. estuarius* to ammonia in both a 96-h "water- only" test and a 10-day "spiked sediment" test. In each instance, LC50s were calculated and expressed based on both measured total ammonia and calculated (Bower and Bidwell, 1978) un-ionized ammonia concentrations. Values derived for these tests are shown in Table F.1. Results for the respective values (i.e., as total ammonia or un-ionized ammonia) indicate somewhat greater tolerance of *E. estuarius* to ammonia in the 96-h "water-only" test, relative to that in the "spiked-sediment" test.

Tay *et al.* (1998) reported a 96-h "water-only" LC50 for total ammonia of 156 mg N/L. This value does not differ markedly from the 96-h water-only LC50 for total ammonia of 104 mg N/L determined for this species by Kohn *et al.* (1994), as well as that (i.e., 144 mg N/L) reported by Bailey *et al.* (1997). The 96-h LC50 of 2.2 mg N/L for un-ionized ammonia calculated by Tay *et al.* (1998) for *E. estuarius* is very similar to that (i.e., 2.1 mg N/L) calculated by Kohn *et al.* (1994), although somewhat higher than that (i.e., 0.8 mg N/L) determined by Bailey *et al.* (1997).

USEPA (1994a) presents *E. estuarius* application limits for both total ammonia and un-ionized ammonia in sediment. These values, identified as "water column no-effect concentrations", are <60 mg NH_3/L (= <49.4 mg N/L) as total ammonia, and <0.8 mg NH_3/L (= <0.7 mg N/L) as un-ionized

No application limits are imposed here for total or un-ionized ammonia in test materials (see Table F.1), inasmuch as the ammonia concentrations in samples under investigation might be elevated due to anthropogenic and/or natural causes, and might be an integral toxic component deserving of consideration using this species and test method.

Tolerance Limits for Hydrogen Sulphide

Hydrogen sulphide can be elevated in sediment porewater to levels toxic to amphipods and other benthic life (Sims and Moore, 1995b). Based on a literature review of measured porewater concentrations of hydrogen sulphide and its known toxicity to marine or freshwater organisms, these authors concluded "*The comparison of reported exposure and effects concentrations suggests a strong potential for hydrogen sulphide toxicity in dredged material bioassays".* To date, however, no definitive data are available showing the limits of hydrogen sulphide in porewater or overlying water (i.e., results of "water-only" LC50s) that *E. estuarius* can tolerate.

Historical Control Performance

Canadian and U.S. laboratory personnel have undertaken numerous 10-day sediment toxicity tests and 96-h *water only* reference toxicity tests with this species. Mean 10-day survival rates in control sediment have routinely been ≥90% in the majority of instances. Additionally, the *water only* controls for reference toxicity tests have typically achieved ≥90% survival during 96-h exposures. Accordingly, minimum mean 10-day survival rates of ≥90% in control sediment, and 96-h survival rates of ≥90% in the control/dilution water used in reference toxicity tests, are considered to be readily achievable and suitable limits on which to base criteria for valid sediment and reference toxicity tests using *E. estuarius* (see Sections 4.6 and 5).

Appendix G

Amphiporeia virginiana — Known Tolerance and Application Limits

Tolerance Limits for Reference Toxicant

Since 1991, Environment Canada's Atlantic regional laboratory has been undertaking "water-only" 96-h LC50 reference toxicity tests with each group of field-collected *Amphiporeia virginiana* used in 10-day sediment toxicity tests. Results for these tests (n = 18), performed at 10°C according to Environment Canada (1992), have been plotted and summarized as warning limits (geometric mean ± 2 SD; Doe, 1997). These summary values (Table G.1) should help guide inexperienced laboratories in selecting an appropriate range of test concentrations for undertaking reference toxicity tests with this species; and are useful for comparative purposes. No reports are available which show the tolerance of *A. virginiana* to cadmium in 96-h "water-only" LC50s performed at other laboratories.

Tolerance and Application Limits for Salinity

Wade and Doe (1992) investigated the tolerance of *A. virginiana* to differing salinities, in a series of 10-day "water-only" survival tests. Mean 10-day survival was 100% at test salinities averaging 30‰ and 25‰, and 80% at salinities averaging 20‰ and 15‰. Only two of ten animals (20%) survived a 10-day exposure to 11‰ salinity; all amphipods exposed to mean salinities of 5‰ or 0‰ died during the 10-day test. *A. viginiana* has also been collected from sites where the salinity of sediment pore water was as high as 35‰ (Doe and Jackman, 1998). No other studies are available which report the salinity tolerance of this species.

Based on the findings of Wade and Doe (1992) and Doe and Jackman (1998), it is evident that *A. virginiana* is tolerant of salinities ranging from 15 to 35‰, and intolerant of salinities <15‰.

An *A. virginiana* application limit of 15 to 35‰ is specified here for porewater salinity (see Table G.1 and Section 4.6). Test material with porewater salinity less than 15‰ must be evaluated for toxicity using another suitable species which is more tolerant of low-salinity water (e.g., *Eohaustorius estuarius;* see Appendix F).

Tolerance Limits for High Organic Content

No studies with formulated sediments are available which demonstrate the effect of elevated levels of organic carbon on the 10-day survival rate for this species. Results for tests with field-collected reference sediments are also not enlightening; no reports are available showing 10-day survival rates for *A. virginiana* when exposed to samples with organic content greater than 2%. Studies with commercial formulations of silica sand (Tay *et al.*, 1998) demonstrate that *A. virginiana* can survive well for 10 days in the absence of any appreciable organic carbon content.

Table G.1 Known Tolerance and Application Limits for Ten-day Tests for Sediment Toxicity Using *Amphiporeia virginiana*

Parameter	Known Tolerance Limits	Application Limits
96-h "water-only" LC50 for reference toxicant (mg Cd/L)	2.0 (0.9 to 4.9) [a]	
porewater salinity (‰)	15 to 35	must be 15 to 35
% very coarse-grained sediment [b]		0 to 100 is acceptable
% fines [c]		must be <90
% clay [d]		must be <35
96-h "water-only" LC50, total ammonia (mg N/L)	151 (121 to 181) [e]	
96-h "water-only" LC50, un-ionized ammonia (mg N/L)	1.1 (1.0 to 1.3) [e]	
10-day porewater LC50, total ammonia (mg N/L)	24.6 (21.2 to 28.0) [e]	
10-day porewater LC50, un-ionized ammonia (mg N/L)	0.1 (0.1 to 0.2) [e]	
porewater hydrogen sulphide (mg/L)	not available	

[a] Geometric mean (± 2 SD) for 18 tests performed at Environment Canada's Atlantic regional laboratory.
[b] Percentage of particles in test material >1.0 mm.
[c] Percentage of particles in test material <0.063 mm (i.e., % silt and clay).
[d] Percentage of particles in test material <0.004 mm.
[e] LC50 with 95% confidence limits in parentheses; based on measured (total ammonia-nitrogen) and calculated-from- measured (un-ionized ammonia-nitrogen; Bower and Bidwell, 1978) concentrations. From Tay *et al.* (1998).

It is known that *A. virginiana* can tolerate samples of test material with a total organic carbon content of 2% or less; however, the upper limit that can be tolerated, without affecting 10-day survival, is not known. No application limit for total organic carbon seems necessary or appropriate.

Tolerance and Application Limits for Grain Size

Studies by Tay *et* al. (1998) have demonstrated that A. *virginiana* is very tolerant of coarse-grained sediment. In tests with differing formulations of silica sand, Tay *et al.* (1998) found a mean 10-day survival rate of 94% for a sample comprised of ~92% very coarse-grained (i.e., particles >1.0 mm) sediment. This species also survived well (mean 10-day survival, 95%) when held in 96.5% coarse-grained (i.e. particles > 0.25 mm) material (Tay *et al.*, 1998). Given the high tolerance of this species to coarse-grained material, no *A. virginiana* application limit for very coarse-grained (i.e., >1.0 mm) material is necessary or appropriate (see Section 2.6).

Tests with both commercial formulations of fine-grained material and fine-grained field-collected sediment indicate that this species is intolerant of a high percentage of fines (i.e., particles <0.063 mm). Tay *et al.* (1998) found 10-day mean survival rates of ≤66% for replicate groups of *A. virginiana* held in commercial sand:silt:clay formulations comprised of ≥36% clay and 50 to 99% fines. Additionally, Tay *et al.* (1998) reported a 10-day mean survival rate of only 56% for replicate groups held in a field-collected reference sediment with 17% clay and 82% fines. Tests with field-collected sediment have demonstrated a somewhat greater tolerance of this species to certain samples with a high percentage of fines and/or a high percentage of clay (Doe, 1998). In consideration of all available data showing mean 10-day survival rates in formulated and field-collected sediment with differing but high percentages of fines and/or clay, and in keeping with the subsequent recommendation by Doe (1998), an *A. virginiana* application limit of <90% fines is designated here (see Table G.1 and Section 2.6). Additionally, a second application limit of <35% clay seems reasonable, and is to be applied as part of this reference method. Accordingly, test material with ≥90% fines and/or ≥35% clay content must not be used for a 10-day sediment toxicity test with *A. virginiana* according to this reference method. Rather, another test species more tolerant of fine-grained sediment (e.g., *Eohaustorius estuarius*; see Appendix F) should be used, provided that grain size characteristics are within the application limits for this species.

Tolerance Limits for Ammonia

Sims and Moore (1995a) undertook a literature review for concentrations of ammonia in sediment pore water, as well as for known toxicity of ammonia to marine and freshwater invertebrates and fish. These authors concluded "*The comparison of reported exposure and effects concentrations suggests significant potential for ammonia toxicity in dredged material bioassays".*

Tay *et al.* (1998) measured the tolerance of *A. virginiana* to ammonia in both a 96-h "water-only" test and a 10-day "spiked sediment" test. In each instance, LC50s were calculated and expressed based on both measured total ammonia and calculated (Bower and Bidwell, 1978) un-ionized ammonia concentrations. Values derived for these tests are shown in Table G.1. Results show an appreciably greater tolerance of this species to ammonia (as total ammonia or un-

ionized ammonia) in the 96-h "water-only" test, relative to that measured in the 10-day "spiked sediment/porewater ammonia" test (see Table G.1). This finding is inconsistent with those for *R. abronius*, *E. washingtonianus*, or *E. estuarius*, in which instances the tolerance of each of these species to ammonia in 96-h "water-only" and 10-day "spiked-sediment" tests was similar (see Appendices D, E, and F). No other studies are available which demonstrate the acute lethal tolerance of *A. virginiana* to ammonia.

No application limits are imposed here for total or un-ionized ammonia in test materials (see Table G.1), inasmuch as the ammonia concentrations in samples under investigation might be elevated due to anthropogenic and/or natural causes, and might be an integral toxic component deserving of consideration using this species and test method.

Tolerance Limits for Hydrogen Sulphide

Hydrogen sulphide can be elevated in sediment pore water to levels toxic to amphipods and other benthic life (Sims and Moore, 1995b). Based on a literature review of measured porewater concentrations of hydrogen sulphide and its known toxicity to marine or freshwater organisms, these authors concluded "*The comparison of reported exposure and effects concentrations suggests a strong potential for hydrogen sulphide toxicity in dredged material bioassays".* To date, however, no definitive data are available showing the limits of hydrogen sulphide in pore water or overlying water (i.e., results of "water-only" LC50s) that *A. virginiana* can tolerate.

Historical Control Performance

Environment Canada's Atlantic regional laboratory has completed 32 separate series of 10-day sediment toxicity tests with *A. virginiana* since March 1991. Mean 10-day survival rates in control sediment averaged 89.5%. Fourteen of the 32 tests (=44%) failed to achieve $\geq$90% control survival; and seven of the 32 tests (= 22%) failed to achieve $\geq$85% control survival. However, 29 of the 32 tests (= 91%) achieved $\geq$80% control survival (Doe and Jackman, 1998). Given this historical control performance, a minimum mean 10-day survival rate of $\geq$80% in control sediment is considered to be a readily achievable and suitable limit on which to base a criterion for a valid sediment toxicity test using *A. virginiana* (see Section 4.6).

In conjunction with the sediment toxicity tests using this species, Environment Canada's Atlantic regional personnel have undertaken 27 separate 96-h *water only* reference toxicity tests with *A. virginiana* since June 1991. For these tests, the mean overall survival rate for the control groups was 97%. Twenty-two of the 27 tests (= 81%) had $\geq$95% control survival, and only one of the tests (= 3.7%) had <90% survival (Doe and Jackman, 1998). Given this historical control performance, a minimum 96-h survival rate of $\geq$90% is considered to be a readily achievable and suitable limit on which to base a validity criterion for a *water only* reference toxicity test using *A. virginiana* (Section 5).