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Hibernia Oil and Gas Production and Development Drilling Project Environmental Effects Monitoring (EEM) Plan

File No. TA1978311

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EXECUTIVE SUMMARY

As part of the planning process for the production phase of the Hibernia offshore oil project, and in accordance with one of the conditions of project approval established by the Canada-Newfoundland and Labrador Offshore Petroleum Board (C-NLOPB), Hibernia Management and Development Company Ltd. (HMDC) has developed an Environmental Effects Monitoring (EEM) Program. The goal of the EEM Program is to assess the effects of Hibernia production and drilling operations on the marine environment.

The EEM program provides the means by which project-induced changes in the surrounding receiving environment are assessed and quantified. The Hibernia EEM program builds upon the earlier stages of environmental planning, including the results of the 1994 baseline EEM survey, which was submitted in 1995 (JWE 1995). The Hibernia Development Project Production Phase Environmental Effects Monitoring Plan (HMDC 2002) was updated in June 2002 to reflect changes and modifications to the Hibernia EEM program that have been incorporated since 1996. It was updated in 2013 (HMDC 2013) to incorporate monitoring related to the Hibernia Southern Extension (HSE) subsea development and is being updated again in 2019 to incorporate monitoring at the Hibernia West Tieback (HWT); the second subsea development at Hibernia.

The design of EEM programs is an iterative process in which there is opportunity to review the design on an ongoing basis over the life of the project to address project changes, address regulatory revisions, and to reflect and incorporate new technologies and methodologies that are deemed necessary. Operational modifications on Hibernia drilling operations in 2001 and 2002 have significantly changed the environmental aspects of the project. The net result has been a clearly identifiable improvement in the receiving environment surrounding the Hibernia production Platform.

Partial reinjection of synthetic-based mud (SBM) drilling wastes commenced in March 2001, with greater than 95 percent reinjection capability achieved in September 2002. This operational changes instituted by Hibernia, coupled with the results since that time indicated that a review of the Hibernia EEM program design was timely and relevant. This review has resulted in revisions and refinements that are contained within this document and builds upon the Hibernia Development Project Production Phase Environmental Effects Monitoring Plan (HDMC 2013).

In Decision Report 2010.02, the C-NLOPB approved an application for an amendment to the Hibernia Development Plan, which included amending the environmental effects monitoring (EEM) plan to incorporate drilling and production activities associated with the Hibernia Southern Extension (HSE) drill centre.

In 2018-19 Hibernia initiated the HWT; this revision of the EEM plan is largely driven by the requirement to monitor potential affect arising from this expansion.

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1.0 INTRODUCTION

1.1 Project Setting

Hibernia is located near the northeast corner of the Grand Banks, approximately 315 km east-southeast of St. John's, Newfoundland, and approximately 35 km northwest of the Terra Nova Oil Field. The White Rose site is located approximately 50 km east-northeast of the Hibernia Platform (Figure 1.1).

The Hibernia field was discovered in late 1979. Following construction of the Hibernia Platform at Bull Arm, Newfoundland and Labrador, from 1990 to 1997, tow out to the Grand Banks occurred in May 1997. The Platform was installed on the seafloor and drilling commenced in June 1997. Throughout the summer of 1997, additional subsea infrastructure (the Offshore Loading System (OLS)) was installed. First oil occurred on November 17, 1997.

Hibernia uses a single fixed Platform, or gravity-based structure (GBS), to complete drilling of at least 64 wells that will be required during the life of the project. In 2013, drilling commenced at the first subsea development; the Hibernia Southern Extension (HSE) which is located approximately 7 km south of the GBS and consists of a subsea water injection flow line running from the GBS to the excavated drill centre (EDC) where water is injected via a manifold into several water injection wells. The subsea wells were drilled using a mobile offshore drilling unit or MODU. An additional subsea tieback, Hibernia Western Tieback (HWT), located approximately 5 km west-southwest of the GBS, is planned for construction (ie. dredging) starting in 2021.

Regulated discharges to the marine environment during production and drilling operations on the GBS include liquid discharges (produced water, storage displacement water, platform drainage water, seawater return and sanitary and domestic wastes), drill cuttings and drilling muds, as well as other approved discharges that may occur on occasion. The locations of discharge points on the GBS for regulated discharges are shown in Figures 1.2 and 1.3. Regulated discharges associated with the MODU (used previously for HSE and to be used for HWT) include water based drill mud and water and synthetic oil based cuttings, bilge water cooling water, as well as sanitary and domestic waste.

On average, water-based muds (WBMs) are used for the first 300 to 400 m of the well depths, and the remainder of wells are drilled using a synthetic-based mud (SBM) system due to challenges posed in extended reach and directional drilling.

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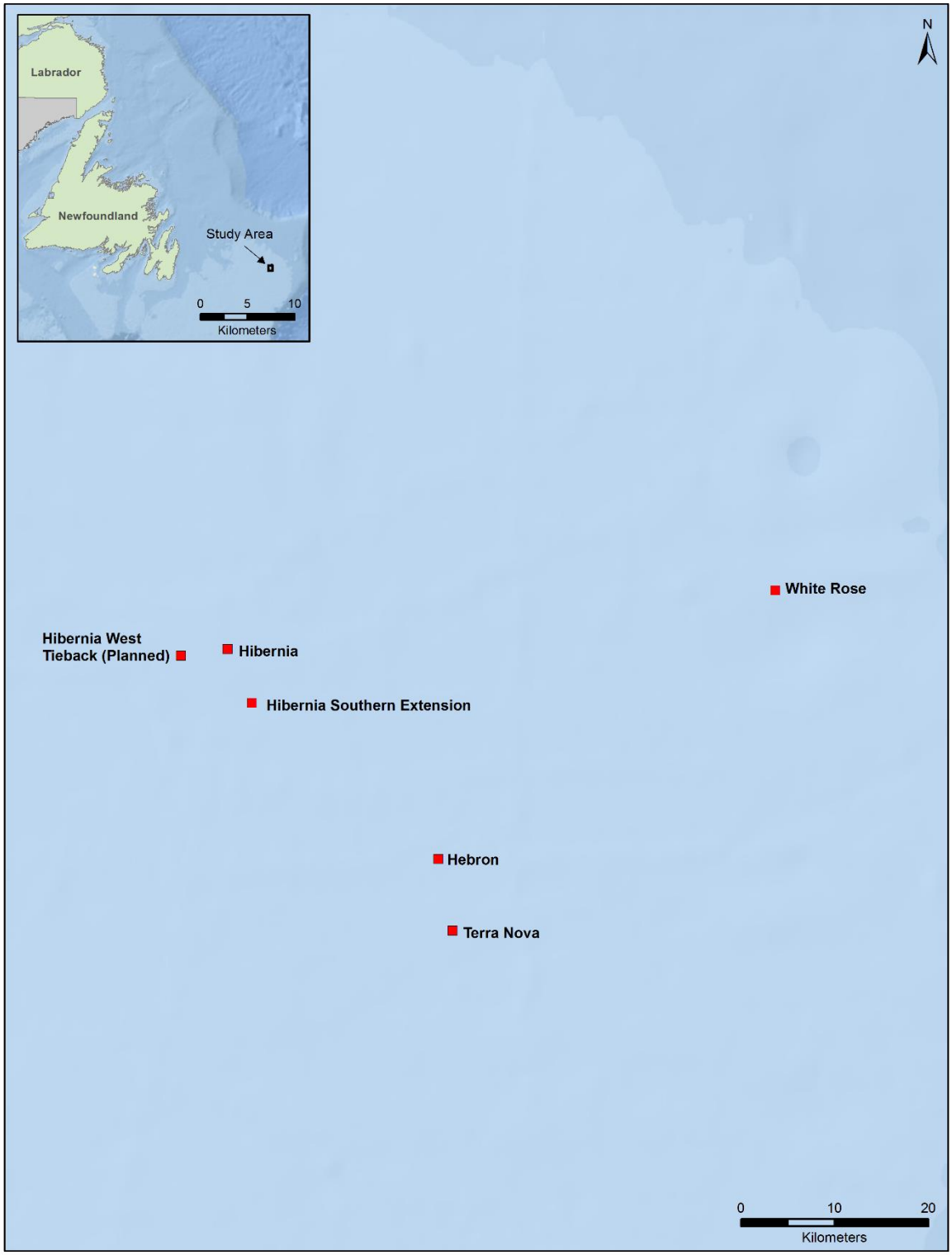

Figure 1-1 Location of the Hibernia Oilfield

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1.2 Project Commitments

In the Environmental Impact Statement (EIS) prepared for the Hibernia project (Mobil 1985), a commitment was made by the Hibernia partners to undertake environmental effects monitoring (EEM). In submitting its Development Plan for the Hibernia project in 1985, Mobil Oil, on behalf of Hibernia, stated:

> *"Effects monitoring will be undertaken to detect changes in the environment surrounding the project that can be attributed to the project."*

The Canada-Newfoundland Offshore Petroleum Board (C-NOPB) approval of the Hibernia Development Plan was announced in June 1986. This project approval was subject to a number of conditions, including:

> **Condition 12**
>
> *"It is a condition of the approval of the Hibernia Development Plan that prior to production, the Proponent submit, for the Board's approval, its plans for environmental compliance and effects monitoring programs."*

The EEM Plan has been through several revisions over the lifetime of the project with the initial approval of the EEM Plan obtained in June 1996 and the most recent revision approved in 2013 with the addition of HSE. This current revision includes the addition of sampling related to the planned HWT and incorporates the lessons learned from EEM results to date.

In 2009, the Hibernia Drill Centres Construction and Operations Program Environmental Assessment determined no likely significant adverse environmental effects with the application of appropriate mitigations. This included construction, operation, maintenance, and decommissioning of up to six excavated drill centres including HSE and HWT for which dredging is planned for 2021. To reduce the potential for adverse effects not being detected, the existing Hibernia environmental effects monitoring program requires amendments to include the effects monitoring of the drill centres as spatially and temporally appropriate. The plan amendment was approved by the C-NLOPB in 2013 for HSE.

The HWT will be the second subsea development and will develop up to seven fault blocks in the south-western sector of the Ben Nevis Avalon reservoir of the Hibernia field. The HWT will be excavated to sufficient depth to provide protection from iceberg scour and associated subsea infrastructure. The current concept for HWT is for construction (dredging) to start in 2021 and key platform modifications are planned to be completed during the planned platform shut-down targeted for the 3rd Quarter of 2021. Installation of flowlines and subsea equipment at the drill centre is scheduled for 2022 and drilling beginning late that same year.

Details on the proposed revisions to the EEM program are presented in this document and builds upon the existing EEM program, using the same methods, analyses and interpretation philosophy.

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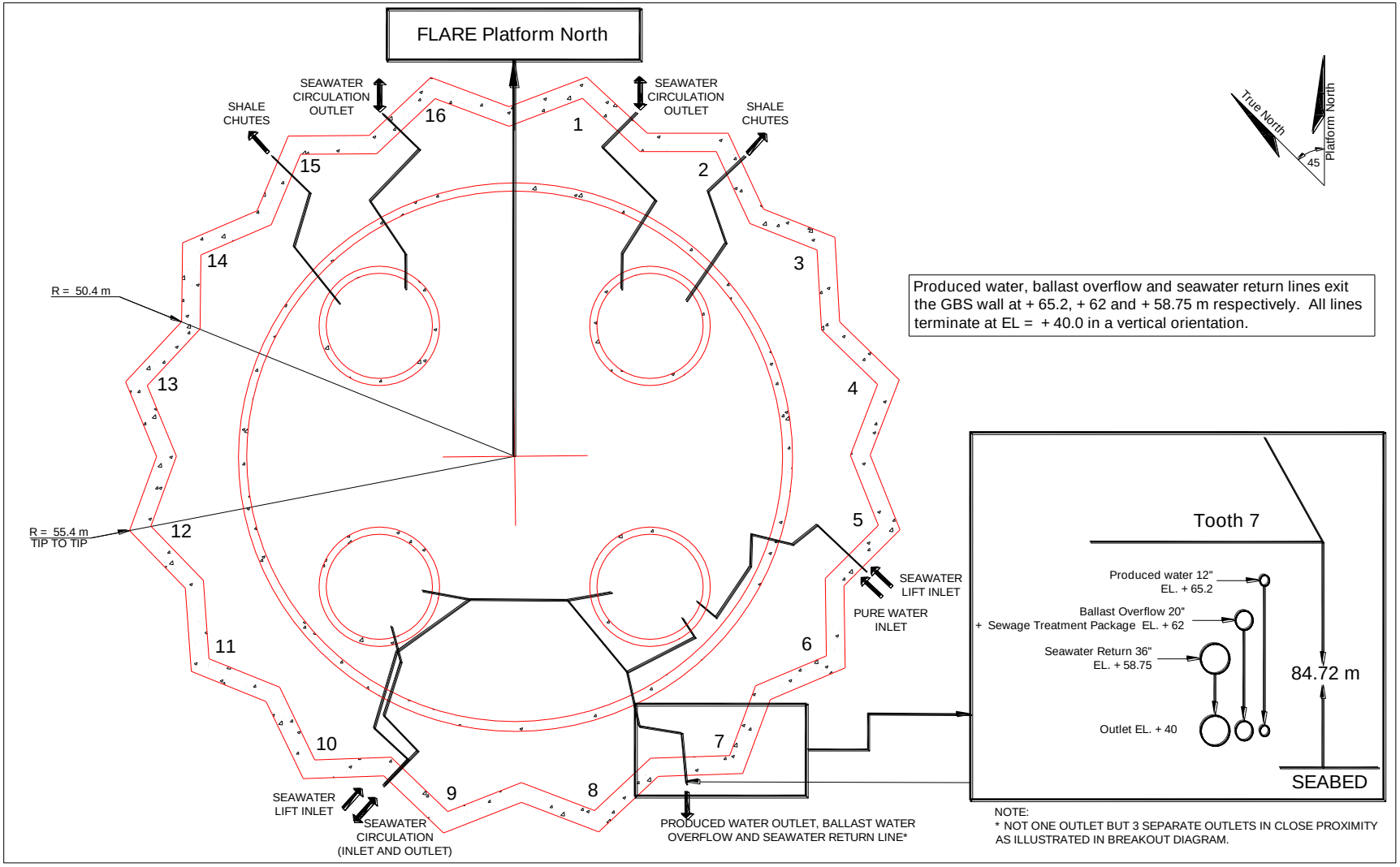

Figure 1-2 Hibernia Platform Cross-sectional Seawater Inlet and Discharge Locations

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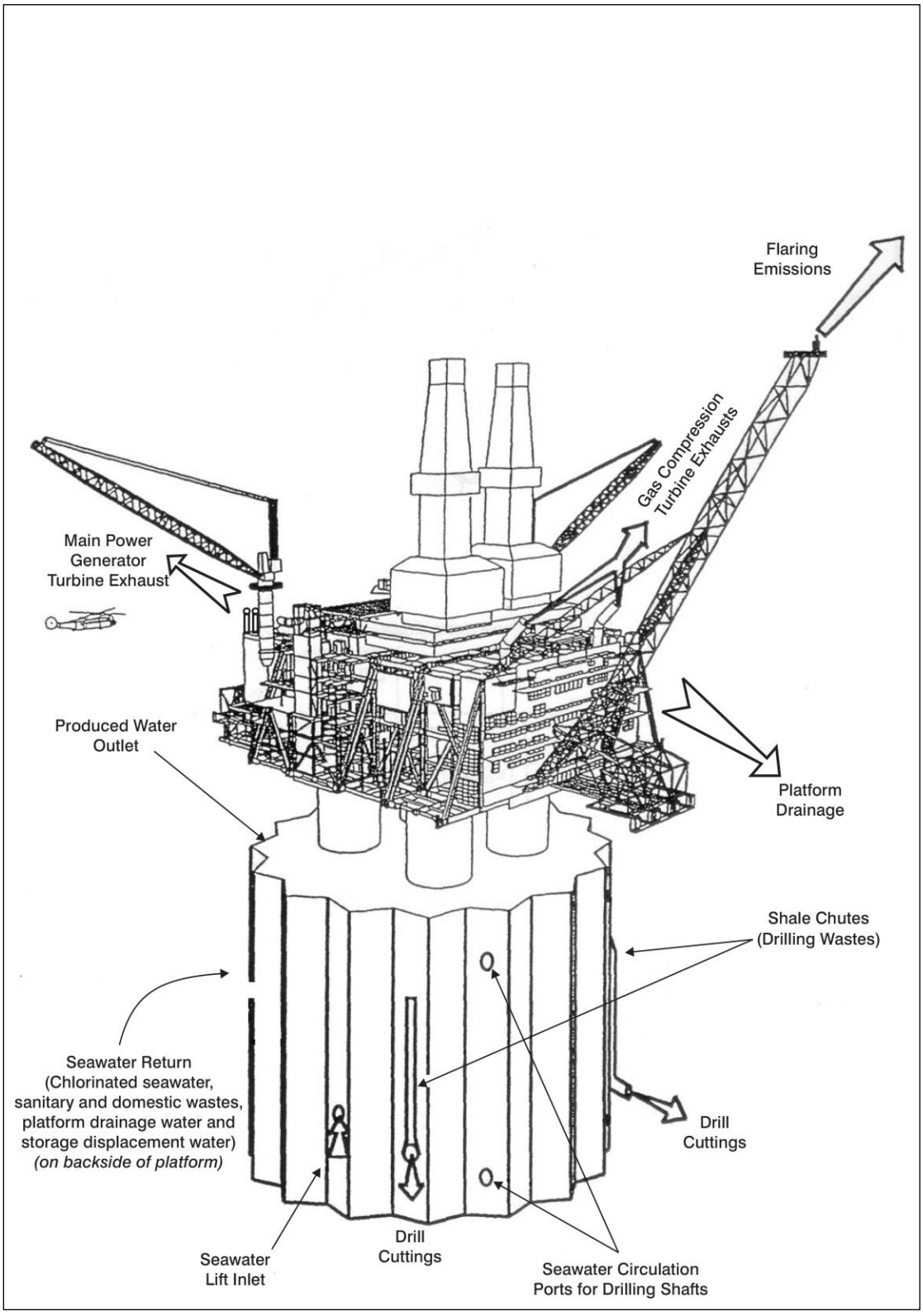

Figure 1-3 Hibernia Platform Seawater Inlet and Discharge Locations

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The planned development of HWT necessitates the update of the EEM program to monitor and measure potential environmental effects associated with the HWT prior to any construction. The HWT EEM program is integrated into this plan and builds upon the existing core EEM program and largely duplicates the HSE sampling, using the same methods, analyses and interpretation philosophy.

The EEM program is one of a series of environmental protection initiatives outlined in the HMDC Operational Plan, which forms an integral part of production operations, including environmental compliance monitoring and emergency response management. Environmental compliance monitoring characterizes effluent to ensure conformance to the discharge limits adopted in the project Environmental Protection Plan(s).

The EEM program is a sampling, analysis and reporting program which is intended to detect effects in the receiving environment resulting from project activities. In addition, it serves to confirm the effectiveness of discharge limits adopted in the project Environmental Protection Plan(s).

1.3 EEM Program Objectives

HMDC established a set of specific objectives to be met in the development and application of its EEM plan (HMDC 1996). These objectives are:

- fulfill regulatory information requirements and address legitimate public concerns;
- provide early warning of potential project-induced environmental effects;
- meet the Project needs;
- be scientifically defensible;
- be cost-effective, making optimal use of personnel, technology and equipment;
- use the data that will be collected for assessment and, where necessary, to modify operational practices and procedures; and
- analyze and interpret data so that the results are understandable both to the public and non-scientists.

The United Nations Group of Experts on Scientific Aspects of Marine Environmental Protection (GESAMP) recognizes the term contamination to refer to elevated levels of a chemical as compared to background levels (GESAMP 1993). They also recognize the term pollution to refer to the effects of the contamination on the biota. The term contamination and the derivation contaminant will be used as per the GESAMP definition in Hibernia's effect monitoring program. The definitions for contamination and pollution as recognized by GESAMP (1993) clearly demonstrate that contamination (i.e., levels elevated as compared to background levels) does not necessarily indicate that an ecological effect has occurred.

Biological effects on marine life would be the principle concern associated with platform effluent discharge. Such effects may be either biological or physical in nature and can be direct or indirect. For example, biological effects may occur when marine life is exposed to contaminated water or sediment, however the magnitude of the resulting effect is dependent upon such factors as the

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nature of the contaminant, its concentration in the marine environment, and the degree of exposure (i.e., related to dose/response).

The EEM program is designed to provide the primary means to determine and quantify project-induced changes in the surrounding environment as it relates to normal operations. Where such changes occur, the EEM program results will provide the basis to determine if environmental assessment predictions are accurate. Where EEM program results contradict environmental assessment predictions, the results will be used to modify this EEM plan and may be used to assist in identifying operational modifications or mitigations when effects are considered significant and unacceptable.

In the event of a large oil spill, an Oil Spill EEM that uses similar monitoring tools will be implemented over the potentially affected area.

1.4 Hibernia EEM Programs and Changes

This section includes a catalogue of EEM programs to date, changes that have occurred over the life of the EEM Plan, and changes that will be implemented going forward.

1.4.1 Hibernia EEM Programs to Date

Table 1-1 lists the previous EEM cruises for Hibernia and HSE. For both Hibernia and HSE EEM Programs, baseline surveys were conducted prior to the initiation of drilling operations in 1994 and 2011, respectively. Subsequent EEM surveys were conducted on an annual basis for the first three years and every two years thereafter with one exception; in 2014 the Hibernia Platform EEM survey was postponed by a year to synchronize with the HSE EEM schedule. A similar requirement to synchronize HWT monitoring with monitoring at the platform and at HSE in 2023 is reflected in the schedule found in Section 6.3.

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Table 1-1: Hibernia EEM Program Sediment and Biological Survey Schedules

Phase	Year	Sediment Cruise Dates[1]	Biological Cruise Dates
Baseline (Hibernia)	1994	Aug. 31-Sep. 10	Dec. 04-06
Year 1 (Hibernia)	1998[2]	Aug. 26-Sep. 01	Dec. 16-23
Year 2 (Hibernia)	1999[3]	Jul. 25-30, Sep. 01-Oct. 04	Jun. 08-10
Year 3 (Hibernia)	2000	Jul. 8-18	Jul. 05-06
Year 4 (Hibernia)	2002	Jul. 06-14	Jun. 29-01
Year 5 (Hibernia)	2004	Aug. 22-28	Jul. 13-14
Year 6(Hibernia)	2007	Aug. 17-29	Jul. 17-20
Year 7 (Hibernia))	2009	Jul. 30-Aug. 04	Jun. 30-Jul. 03
Year 8 (Hibernia) Baseline (HSE)	2011	Aug. 01-06	Jul. 07-12
Year 9 (Hibernia) Year 1 (HSE)	2014[4]	Aug. 07-17	Jun. 20-Jul. 01
Year 2 (HSE)	2015	Sep. 10-16	Jun. 28-30
Year 10 (Hibernia) Year 3 (HSE)	2016	Aug. 30-Sep. 6	Jun. 25-Jul. 04
Year 11 (Hibernia) Year 4 (HSE)	2018	Jul. 19-Jul. 30	Jun. 29-Jul. 06

Notes:
[1] The sediment cruise for Hibernia sampling also includes water sampling
[2] The 1999 sediment cruise was split into two cruises due to equipment losses and manufacturing of new equipment
[3] Hibernia began production in November of 1997
[4] Drilling at HSE commenced in January 2014 (HMDC, 2017)

1.4.2 Hibernia EEM Changes

The Hibernia Development Project Production Phase Environmental Effects Monitoring Plan (HMDC 1996) built upon data and information collected during the Hibernia Baseline EEM Program (HMDC 1995). The development and design of the Hibernia Baseline EEM Program was finalized after extensive consultation with regulatory agencies and regional, national and international experts in the offshore oil EEM programs.

The design of an EEM program depends upon the specific objectives of the program. Program objectives can change over time in response to evolving knowledge about the project, and its potential or observed interactions with the environment. Therefore, it is reasonable that the EEM program design should evolve or change over time.

The major modifications that have been incorporated to the Hibernia EEM program are provided in Table 1-2. In previous Hibernia EEM surveys (up to 2002), a high level of emphasis was placed upon replication at each survey station but has since been determined to not be necessary for the primary statistical analysis. Therefore, a reduction in the level of replication for post-2002 EEM programs has been implemented (one grab per station) with a 10 percent level of replication for QA/QC purposes.

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Table 1-2: Previous Major Modifications for Hibernia EEM Program

Year	Hibernia EEM Program Modifications
1994	• Benthic habitat assessments removed. • Suite of sediment toxicity bioassays added.
1998	• Echinoid fertilization bioassays removed. • Juvenile polychaete growth bioassays added.
1999	• More intense sampling conducted within 1000 m (sampling along all 8 radials at 500, 750 and 1000 m conducted resulting in the addition of 12 stations). • Iceland scallops removed due to absence in area • Biological survey changed from winter to summer sampling.
2000	• 32 Km stations added along radial 1 and 7
2002	• 32 km stations discontinued. • Chemical analyses on sediment for sulphides and ammonia added. • Fish health pilot program conducted.
2004	• Sediment sample grid revised focusing on near field stations and limited to 4 radials outside of 1000 m. • Replicate sampling at each station (pre 2004 there were 3 samples per station) discontinued. • Fish health added as a permanent component of Hibernia EEM program. • Sampling focused on select metals and TPH hydrocarbons in sediment. • Pilot water quality program conducted.
2007	• Water quality program added as a permanent component of Hibernia EEM program. • Advanced statistical analyses conducted on parameters only when differences between baseline, 2002 and 2004 data are detected. • Water quality sampling to be conducted at fixed stations at 50, 100 and 200 m from produced water discharge. • Three samples are to be collected per water column station (one from near surface, one from within the plume location and one near bottom). • Due to the fluid nature of the produced water plume, predetermined sample locations within 50 m of the discharge point should not be imposed, rather these sample locations be directed by the CTD Profiles. • When logistically feasible, produced water sampling (including toxicity testing) for Environmental Protection Plan be conducted concurrently with the water column program.
2009	• Due to the fluid nature of the produced water plume, predetermined water quality sample locations within 50 metres of the discharge point not imposed. Sample locations for each program year be directed by the CTD profiles and safety risk management.
2011	• Baseline effects monitoring data collected for Hibernia Southern Extension subsea development
2013	• Revised to incorporate HSE drill centre

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Year	Hibernia EEM Program Modifications
2019	• Revised to incorporate HWT drill centre • Alkyl PAHS removed from sediment analysis and fish tissue analysis • Replace Microtox test with Petrotox sediment quality guideline as screening tool for all stations for further toxicity testing • Change species for Juvenile polychaete toxicity test • Increase separation distance between sampling locations and the GBS, subsea equipment and hazards. (100m for sediments, 50m for water and 1000m for fish). • Commercial fishing field level focused not focused on assets within the field. • Sample archiving changed to 12 months or one month after raw data submitted to C-NLOPB whichever is later • Increase in reference area size

Key changes that have been incorporated into this version of the EEM design plan are described in Table 1-3 with full discussion and rationalization detailed in the relevant sections.

Table 1-3: Current Modifications for Hibernia EEM Program

Component	Details	Section Discussed
Sediment Quality	Additional 12 Sediment stations located around the HWT drill centre	3.0
	Removed alky PAHs from sediment quality analysis	3.1.1
	Samples to be archived for 12 months or one month after results are submitted to the CNLOPB whichever is later	3.1.1
	Replace Microtox test with Petrotox sediment quality guideline as screening tool for further toxicity testing.	3.1.2
	Petrotox sediment quality guideline to be applied to all stations including those inside 500m	3.1.2
	100m separation distance between sediment sampling sites and subsea equipment	3.2
	Change species for Juvenile polychaete toxicity test	3.1.2
Water Quality	Limit sampling to no closer than 50 m from the GBS	4.3
	CTD no longer necessary to identify plume location	4.6
Commercial Fishing	Commercial Fish sampling now focused at field level not asset level within the field	5.0
	Removed alky PAHs from fish tissue analysis	5.1.1
	Change archive protocol to retain spleen and discard other organs	5.1.3
	Larger Reference Area	5.2
	Fish trawl transects to be located no closer than 1000m to subsea equipment and debris/hazards	5.2
	Increase in reference area size	

1.4.3 Risk Assessment Workshop

Three of the current modifications identified in the previous section were identified as mitigations in an EEM Risk Assessment held on April 30, 2019. The EEM Risk Assessment was held to assess the safety and environmental risks associated with the Hibernia and Hebron EEM programs. The workshop was attended by representatives of HMDC, ExxonMobil Canada, Wood, and Fugro Geosurvey and more than 30 actions were identified to mitigate risks to workers and

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assets. The three actions most relevant to this EEM Plan update are listed in Table 1-4. With the increase in subsea infrastructure in the field, the primary hazard of concern is sampling equipment (ie. box-corer and otter trawl) making contact with subsea infrastructure resulting in a safety and/or environmental incident. The key mitigation identified was to enlarge existing separation distances between subsea infrastructure and sampling areas. The mitigation for hazards associated with the water column sampling is based on potential for vessel collision with the platform. A strong surface current exits immediately adjacent to the platform; this is induced by platform discharges and the associated upwelling effect. This requires the sampling vessel to set thrusters to create a force which pushes the vessel toward the platform to keep position. As the discharge rates vary so does the current creating a potential for the vessel to thrust toward the platform. A rogue wave could also create a similar result. The other risk identified was the potential for the vessel super structure to impact the south lifeboat station. Hibernia's new vessels are much larger and higher than the initial fleet.

Table 1-4. EEM Plan Changes from EEM Risk Assessment

EEM Component	Risk Scenario	Mitigation Revision to EEM plan
Sediment Quality	During deployment of the sediment box corer, it is incorrectly positioned on or dropped on subsea equipment (OLS, SAL bases, flowlines, fiber optic lines, other debris, etc.) resulting in equipment damage to flowline and oil or gas release	100 m buffer for sediment sampling from subsea equipment (previously 50 m)
Water Quality	During deployment or retrieval of the Niskin water sampling kit, vessel makes contact with GBS and/or south lifeboat station causing damage and down manning due to reduced escape capacity.	Limit sampling to no closer than 50 m from the GBS (previously 20-30m)
Commercial Fishing	During trawling, entanglement occurs with subsea infrastructure (OLS, SAL bases, flowlines, fiber optic lines, other debris, etc.) resulting in injury to vessel crew, damage to vessel and/or subsea equipment, and/or environmental incident.	1,000 m buffer between fishing trawl transects and subsea equipment (previously 500m)

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2.0 MONITORING STRATEGY OVERVIEW

The EEM program is designed around the monitoring of the critical elements of the receiving environment that have provided the most beneficial and timeliest information on potential deleterious effects to the receiving environment. It builds upon requests and recommendations provided from regulatory review and experience gained from previous Hibernia EEM programs. Specifically, data will be collected on sediment quality, water column chemistry and a commercial fish species. Data will also be collected on raw produced water to enable verification of predicted dispersion factors.

2.1 Contamination Sources

The Hibernia drilling and production operation and its associated discharges have the potential to affect three major components of the receiving environment:

- Sediment or Seafloor
- Water Column
- Potential Effects on fisheries that could be affected by changes to seafloor or water column

Seafloor - Particulate matter and the associated products associated with production and drilling discharges is predicted to settle out around the Hibernia Platform, as modelled by Seaconsult (1994), with chemical constituents being chemically sorbet by particulate matter. This net accumulation in the sediment may alter the sediment toxicity over time and can be incorporated into the food web via bottom-feeding fish and filter-feeding bivalves. Sampling and analysis of the sediments surrounding the Hibernia assets (ie. GBS, HSE, and HWT) provide a mechanism for determining the trend in change in concentrations that could be associated with biological effects due to operational discharges. Sediment toxicity assays have been used to assess the biological significance of project related contamination in sediment samples collected from the receiving environment adjacent to the Hibernia platform since 1994. The rationale of this approach is non-toxic sediments are able to support diverse and abundant benthic communities, whereas toxic sediments are unable. Synthetic based mud (SBM) and cuttings re-injection (CRI) equipment was installed and commissioned in 2001, with essentially all SBM drill cuttings and solids from the platform being reinjected into the reservoir by fourth quarter 2002 and no longer fully discharged. Limited discharges of SBM cuttings was initiated in 2015 for certain situations (ie. casing shoe tracks, cement plugs) to ensure the integrity of the CRI system. The majority of SBM cuttings, however, continue to be reinjected via the CRI system. Thus, the primary source of synthetic-based fluid at the Hibernia platform is the two cuttings piles located below the shale chutes reflected in Figure 1-3. Unlike the platform, SBM cuttings generated on mobile offshore drilling units (MODU) operating at EDC's will continue to be treated and released in accordance with the approved environmental compliance monitoring plan.

Water Column - Produced water is defined as "all water separated from crude oil or gas during the primary processing of oil and gas on offshore production platforms" (Environment Canada

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1990) and consists of both injected seawater and formation water. This definition encompasses formation water, condensed water (from gas compression), injection water (seawater injected to enhance oil recovery) and approved production and injection water treatment chemicals (HMDC 1994). Produced water has elevated levels of total dissolved solids, ammonia and hardness. It contains a variety of hydrocarbon fractions, including selected PAHs. The two primary components of produced water that are of environmental concern are the low molecular weight PAHs (particular naphthalene) and the benzene, toluene, ethylbenzene and xylene (BTEX) fraction of TPH analysis, particularly benzene (Berry and Wells 2004). BTEX is soluble in seawater and highly toxic to marine organisms. PAHs tend to be less soluble but more persistent in the environment (Holdway and Heggie, 2000). Most chemical constituents are rapidly diluted, dispersed, volatized to the atmosphere, absorbed to naturally occurring particles and settled to the bottom, metabolized by bacteria and marine organisms, and are subject to chemical process such as chemical oxidation and photo-oxidation, as well as biodegradation. Metals most frequently found at elevated concentrations in produced water as compared to the receiving environment tend to be barium, iron, manganese, mercury and zinc with Hibernia's produced water having barium, iron and manganese concentrations higher than the receiving environment (Neff et al 2011). Yeats et al. (2011) conducted studies in 2005 and 2006 that indicate particulate barium is the strongest indicator of a produced water tracer at Hibernia.

Commercial Fisheries – Although there are other discharges, the regulators and public have always expressed concern regarding the potential for offshore development projects to produce effects on fish and the fishery. These concerns are based primarily on approved discharges of:

- SBM cuttings;
- Produced water.

As discussed above, the effects associated with SBM cuttings have been significantly reduced at the Hibernia platform due to the achievement of greater than 95 percent cuttings reinjection for typical operations; however, there remains a need of fully understanding the remaining waste streams. Potential environmental effects on biota from these discharges are hydrocarbon and/or trace metal bioaccumulation, tainting and effects on fish health. SBM cuttings were, however, discharged at HSE and will be discharged at HWT once treated in accordance with the approved Environmental Compliance Monitoring Plan.

Table 2-1 summarizes the interactions between the operational discharges and monitoring components.

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Table 2-1: Environmental Effects Monitoring Components

Potential Platform /Environment Interaction	Monitoring Component
Potential for changes to sediment quality. *Seafloor and Sediments serve as sink for platform discharges; most notable drill cuttings*	Analysis of Sediment Chemistry
	Analysis of Sediment Toxicity
Potential changes to Water Quality *Produced Water and other liquid waste discharged into the water column*	Analysis of Water Quality
Potential Effects of Commercial Fish *Potential Effects on Fishery mostly concerning SBM cuttings and produced water*	Analysis of Commercial Fish

2.2 EEM Components

Methods used to assess environmental effects of an activity have evolved from the basic chemical analyses to more exhaustive studies that integrate physical, chemical and biological testing. This constitutes an integrative weight-of-evidence approach to the assessment of environmental effects, examining multiple factors and trophic levels. The integrative assessment or weight-of-evidence approach provides more information on spatial extent, the magnitude of contamination and potential biological effects associated with the contamination than any one single component.

The EEM components and target parameters are summarized in Table 2-2. Details and rationale for each component are provided the Sections 3, 4, and 5 for sediment quality, water quality, and commercial fish, respectively. Any changes to previous iterations of the 2013 plan (summarized in Table 1-3) have been summarized in the introductory section to each component (Sediment Quality, Water Quality, Commercial Fishing) and described further in the supporting text.

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Table 2-2: Environmental Effects Monitoring Components

Component/Matrix	Categories	Specific Parameters
Sediment Quality	Chemistry	General Chemistry (particle size, organic carbon, Sulphur species, redox) Metals Hydrocarbons
	Toxicity	Petrotox Marine Sediment Quality Guideline Juvenile Polychaete Growth Amphipod Toxicity
Water Quality	Chemistry	General Chemistry Metals Hydrocarbons
Commercial Fish	Sensory Evaluation	Taste Tests
	Fish Health	General Morphometrics Stress Enzyme Activity Gill/Liver Histopathology Haematology
	Body Burden	Metals Hydrocarbons

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3.0 SEDIMENT QUALITY

The purpose of sediment surveys is to evaluate changes in sediment characteristics and properties as they may relate to effects of discharges from the Hibernia field development and production on the existing environment of the Grand Banks. The survey can be divided into three elements:

- Contaminant fate assessment associated with the drill cuttings discharge;
- Contaminant effects assessment associated with the drill cuttings discharge; and
- Produced water affects assessment as a results of sedimentation and flocculation.

Data on sediment quality has been collected in each EEM monitoring year since 1994. The major results and trends from over 20 years has been reviewed to improve on the EEM plan. Based on review of results, several changes are reflected in the Sediment Quality portion of this EEM Plan update which includes:

- Reduction in some analytes (i.e. alkyl PAHs) that have a history of undetectable concentrations (see Section 3.1.1)
- Setting an expiry date on all archived sediment samples (see Section 3.1.1)
- Change in the approach to toxicity by using a more reliable toxicity based screening tool (Petrotox sediment quality guideline) to trigger additional toxicity testing and applying that tool to stations ≤ 500m from the platform or drill centres
- Changing species for Juvenile Polychaete Growth (see Section 3.1.2).
- Establish a separation distance of 100 m from sediment sample locations and adjacent subsea assets (see Section 3.2)

Further explanation and rationale for these changes is provided in the referenced sections.

3.1 Sediment Quality Parameters

As demonstrated by the results of the Hibernia EEM program since 2002, the introduction of cuttings reinjection has resulted in improved sediment quality, with continued improvement expected into the future. In part due to the improvements observed in sediment quality, operational changes initiated by Hibernia, and based on recommendations from the 2002 Hibernia EEM program report (HMDC 2002), an in-depth review of the sampling grid design was again initiated in 2003 to determine whether the current sediment design is still relevant and suitable. Based on this review, the sediment sampling grid was revised for core EEM programs.

A review of the sediment chemistry collected since 1994 baseline indicates that the chemical parameters of concern, or those that have been detected as changing over the course of the project, are limited to total petroleum hydrocarbon (TPH) and barium. The focus of the EEM plan and subsequent reporting, therefore, will be on these parameters although other parameters (additional metals, hydrocarbons, and general chemistry) are included and discussed below.

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3.1.1 Chemistry

Metals - The original EEM program design incorporated sediment chemical parameters based upon the PARCOM (1989) guidelines, with appropriate modifications recommended by Dr. S. MacKnight (1994). Table 3-1 identifies the original recommended metals for the program. However, a standard suite of laboratory metal analysis contains a total of 23 metals (which includes the recommended constituents) has been conducted for all EEM programs, as it offered the best value and provided additional information on Grand Banks sediment chemical profiles. The metals data collected since 1994 provide a solid base of information on the geochemical characterization for the Hibernia surface sediments and essentially serves as a baseline that has been collected over an extended period. Although a review of the data indicates that the full suite of metal analyses is not required to assess environmental effects associated with the Hibernia waste streams, the full suite of metals outlined in Table 3-1 will be subject to laboratory analysis in subsequent EEM programs. For completeness, analysis for weak acid leachable barium will also be conducted. Data on metals contained within the standard suite analyzed will be retained and archived for reference only and not subject to statistical analyses or discussion. However, the laboratory data on all metals will be reported in a Data Appendix.

Table 3-1: Sediment Trace Metal Parameters (Total Metals)

Recommended Sediment Trace Metal Parameters (Total Metals)	
Aluminum	**Lithium**
Antimony	Manganese
Arsenic	**Mercury**
Barium*	Molybdenum
Weak Acid Ext. Barium	Nickel
Beryllium	Selenium
Cadmium	Strontium
Chromium	Thallium
Cobalt	Tin
Copper	Uranium
Iron	Vanadium
Lead	**Zinc**
Metals in bold were the original recommended metals (1994) *Barium is the only metal that has shown consistent evidence of project-induced changes	

Hydrocarbons - TPH analysis will continue to be included in the EEM program. There has been no detection of polycyclic aromatic hydrocarbons (PAHs) in marine sediments at Hibernia over the life of the monitoring program. However, in recognition of the risks associated with PAHs as environmental contaminants, non-exhaustive PAH sampling and analyses will be continued. The list of hydrocarbon parameters to be included in hydrocarbon analysis is provided in Table 3-2. Note that alkylated PAHs were added to the sampling design in 2009 but to date have not been detected. They are not part of the standard suite of PAHs and are therefore removed.

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Table 3-2: Sediment Petroleum Hydrocarbon Monitoring Parameters

Sediment Petroleum Hydrocarbon Parameters	
BTEX	
C6-C10 (less BTEX)	
C10-C21 (Fuel Range)[1]	
C21-C32 (Lube Range)	
Polycyclic Aromatic Hydrocarbons (PAHs)[2]	
1-Methylnaphthalene	Chrysene
2-Methylnaphthalene	Dibenzo(a, h)anthracene
Acenaphthene	Dibenzothiophenes
Acenaphthylene	Fluoranthene
Anthracene	Fluorene
Benzo(a)anthracene	Indeno(1,2,3-cd)pyrene
Benzo(a)pyrene	Naphthalene
Benzo(b)fluoranthene	Perylene
Benzo(k)fluoranthene	Phenanthrene
Benzo(e)pyrene	Pyrene
Benzo(ghi)perylene	
Notes: [1]C10-C21 Fuel Range hydrocarbons is the only hydrocarbon analyte in the sediments that has shown consistent evidence of project-induced changes [2]Alkylated PAHs which were added in 2009 are now removed as they have not been detected in the sediment.	

General Chemistry - In addition to metal and hydrocarbon analyses, ancillary parameters that are necessary for sediment data interpretation and integrative assessment will be maintained. These parameters are particle size characterization, organic carbon, inorganic carbon, ammonia and sulphide analyses (Table 3-3).

Table 3-3: General Chemistry Monitoring Parameters

General Chemistry
Sediment Particle Size[1]
Redox Potential[1]
Total Inorganic/Organic Carbon (TIC/TOC)
Sulphur/Sulphide[1]
Ammonia
Notes: [1]Sediment Particle Size (percentage of fines), redox potential, and sulphur/sulphate have shown weak or occasional evidence of project-induced changes.

The parameters listed in Table 3-1, Table 3-2, and Table 3-3 will be sampled at all Hibernia, HSE, and HWT sediment stations. Note that archived samples will no longer be kept indefinitely as in previous versions of the EEM design (HMDC 2013) because most parameters exceed laboratory hold times within six months. Samples will be retained up to 12 months or one month after CNLOPB receipt of the laboratory results whichever comes later.

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3.1.2 Sediment Toxicity

Previously, toxicity assays used for the Hibernia EEM program included three components; Microtox Solid Phase Assay, Amphipod Survival, and Juvenile Polychaete Growth. Microtox testing was performed at all sediment stations. Amphipod Survival and Juvenile Polychaete Growth were performed at all stations within 500 m of the asset (HSE or Hibernia platform) and all other stations that failed the Microtox test outside of 500m were also tested for Amphipod Survival and Juvenile Polychaete Growth.

Reviewing the results to date from Hibernia and HSE and other EEM programs in this area reveals Microtox has not been a good indicator of project induced change (HMDC 2018). Therefore the Petrotox marine sediment quality guideline of 150 mg/kg for C10-C21 (Stantec 2013) will replace Microtox as a screening tool for further Amphipod and Juvenile Polychaete Growth and toxicity testing for all sediment stations including those inside 500 m. The Petrotox guideline was suggested as a replacement for Microtox in the 2016 EEM Monitoring Report (HMDC 2018) with agreement from the C-NLOPB. The proposed changes are outlined in Table 3-4.

Table 3-4: Revised Toxicity Sampling for EEM

Previous Approach	Current Approach
Use Microtox as trigger for further Amphipod Toxicity and Juvenile Polychaete Growth toxicity testing for stations >500m from Hibernia GBS or HSE	Use Petrotox as screening tool for Amphipod Toxicity and Juvenile Polychaete Growth ans Toxicity testing for all sediment stations in the Hibernia field excluding baseline survey in which all stations will be subject to both toxicity tests.
Toxicity sampling includes Microtox, Amphipod Toxicity, and Juvenile Polychaete Growth	Toxicity sampling, Amphipod Toxicity, and Juvenile Polychaete Growth
Pacific species, *Neanthes arenaceodentata,* used for Juvenile Polychaete Growth testing.	Atlantic species, *Polydora cornuta*, used for Juvenile Polychaete Growth testing.

Review of Microtox Results and Comparisons to Petrotox

Microtox results have been variable between survey years and have shown no correlation with sediment chemistry analytes. They have also been inconsistent with other toxicity bioassays. The absence of any degree of correlation makes Microtox a poor candidate as a screening tool for other toxicological assays. Other studies have also described the test as inconclusive (Radovic et al., 2012) and called into question its usefulness within the context of offshore oil and gas environmental monitoring programs (Whiteway et al., 2014).

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For example, at the Hibernia platform in 2016 there were seven toxic responses based on Microtox assay results and none from either the Juvenile Polychaete Growth or Amphipod Survival assays (HMDC 2018). There was no evidence of a significant Project-related effect from Microtox testing from coarse nor fine scale analysis.

To assess replacement of the Microtox test with a more useful toxicity based Petrotox sediment quality guideline, HMDC initiated a detailed assessment of PureDrill IA 35-LV using the Petrotox model (Stantec 2013). Rather than focusing on all chemical alterations in the sediment (which are any level above background), the exercise identified ecologically relevant concentrations of PureDrill IA 35-LV using acute and chronic toxicity test data for constituents of PureDrill IA 35-LV. Threshold concentrations were identified, below which effects would not be predicted (i.e., no observed effect concentration level). The Petrotox threshold equates to biological, rather than chemical thresholds. The no observed effect concentration level obtained via Petrotox was 150 mg/kg, with a range of 115 to 190 mg/kg (Stantec 2013). Based on the 150 mg/kg threshold for PureDrill IA 35-LV and examination of sediment concentration data from the Hibernia EEM since 1994 (HMDC 2018) as well as a review of the three existing EEM programs at that time in the Newfoundland offshore (Stantec 2016), it is concluded that biological effects would be limited to approximately 500 m from the drilling discharge source.

The results from the last round of Hibernia EEM (HMDC 2018) revealed that no toxic responses for amphipod or juvenile toxicity were elicited from any of the stations within 500 m of the platform since 2002 when SBM cutting reinjection was established. Sampling at HSE did have a single station (E-250) with a toxic response for all three current indicators but Microtox was triggered at 11 additional stations without other toxic responses. The Petrotox standard of 150 mg/Kg C10-C21 would have triggered the additional testing at E-250 (Table 3-5).

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Table 3-5: Sediment Toxicity and Petrotox Comparisons for 2016 HSE Results

Station ID	Microtox	Amphipod Survival	Juvenile Polychaete Survival	Petrotox
N-250	X			
N-500	X			X
N-1000	X			
E-250	X	X	X	X
E-500	X			X
E-1000	X			
S-250	X			X
S-500				X
S-1000	X			
W-250	X			
W-500	X			
6000-1	X			
6000-4			X	
Note: X Indicates a positive toxicological response or exceed the Ptox guideline				

Toxicity in the previous versions of the EEM plans was used to measure lethal and sub-lethal effects. Microtox served as a second sublethal toxicity test in addition to Juvenile Polychaete growth. Petrotox would serve that purpose as it was developed based on both lethal and sublethal toxicity values. The null hypothesis testing did not specify the toxicity testing to be used (see Section 7.2.1) and the substitution of Microtox with Petrotox will not reduce the robustness of the EEM program or the statistical design in general.

Application of the Petrotox Sediment Quality Guideline

Based on review of toxicity methods and past results, Microtox testing is being replaced with the Petrotox marine sediment quality guideline (that is stations with C10-C21 concentrations in the sediment greater than 150 mg/kg) as the initial screening, and trigger, for further sediment toxicity testing (i.e., Amphipod Survival and Juvenile Polychaete Growth). The Petrotox guideline will also be used to screen for toxicity testing for stations within 500 m of the discharge site (ie. GBS, HSE, or HWT). Amphipod Survival and Juvenile Polychaete Growth Testing will still occur at Reference Stations and for all baseline sampling stations around planned EDCs like the HWT EDC.

Change to Juvenile Polychaete Species

Environment Canada and Climate Change (ECCC) has recommended that the EEM program switch the current Pacific polychaete species, *Neanthes arenaceodentata*, with *Polydora cornuta*, which is a species found naturally in Canadian (Atlantic) coastal waters (EC 2001). Based on ECCC's recommendations, *P. cornuta* will be used going forward. To assess any differences in toxicity results between the two species, both species will be used for the 2019 EEM baseline

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sampling period however a single species, *P. cornuta*, will be used for subsequent EEM programs assuming the 2019 results do not reveal issues of concern.

3.2 Sediment Quality Sampling Stations

Previous Sediment Sampling Grid Designs (prior to 2004)

The original EEM sample design was a gradient-to-background approach with geometric increase in station distance from the Platform. It was designed to test for the extent and magnitude of chemical occurrence associated with the waste streams from the Platform.

Revisions to previous sediment sampling grids are illustrated in Figure 3-1. The original grid (1994-1998) consisted of eight radials based on compass directions, with a geometric progression of concentric circles selected to represent zones of influences as described in the Seaconsult model (1994). The sample grid design used for 1999, 2000 and 2002 had 56 stations located on the sampling grid within an assumed maximum contaminant migration distance of 8 km and two Reference sites at 16 km on the north and west radials, for a total of 60 stations (Figure 3-1).

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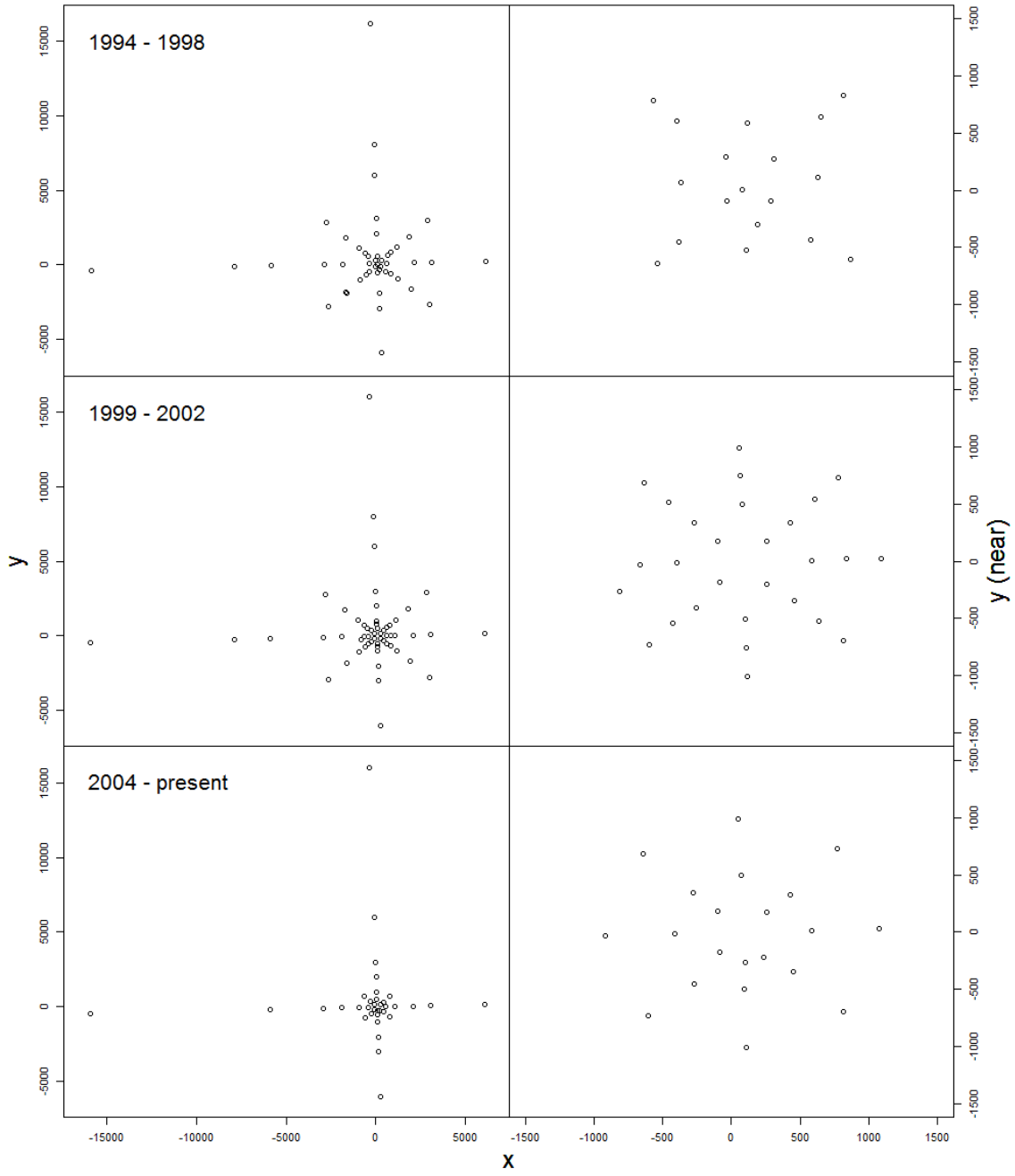

Figure 3-1: Hibernia Sediment Sampling Grids since 1994; left column is out to 6,000 m and right column is out to 1,000 m.

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Changes to Sediment Sampling Grid Design

The current sampling grid design around the Hibernia platform will essentially remain in place (Figure 3-2) with one exception; the separation distance between a station and subsea infrastructure be increased to 100 m. This change will result in a relocation in stations listed in Table 3-6. Most of the stations requiring relocation still retained their nominal distance (i.e, 250 m, 500m, etc.) with the only exception being one station at the GBS (8-250) and one station around HWT (2NE-250); these stations had to be moved to 275 m from the asset to maintain the 100m buffer.

Table 3-6: Sediment Stations Relocated to Enable a 100-m Buffer

HSE[3]	Hibernia	HWT
N-250 W-250 W-500 W-1000	2-250 4-250 5-500 5-2000 6-250 6-500 7-500 7-1000 7-6000 8-250[1] 8-500	2NE-250[2]

[1]Moved station so that it is now 275 m from the platform (i.e., 8-275)
[2]Moved station so it is now 275 m from the drill centre (i.e., 2E-275)
3Note that stations identified in 2013 Plan for HSE were revised in 2014 due to subsea infrastructure

Establishing a 100 m separation distance between sampling locations and subsea equipment was a mitigation identified in the April 30, 2019 Risk Assessment as described above in Section 1.4.3. No stations had to be removed entirely but rather relocated to accommodate a 100-m buffer.

The existing HSE design will largely remain the same as previous EEM programs (Figure 3-3) with the exception of those changes to station locations identified in Table 3-6. The HWT sediment stations are similar in design to HSE with 4 radials (2N, 2S, 3W, 3E) with 3 stations at 250, 500, and 1000 m distances from the drill centres (Figure 3-5). The only exception is that the HWT will not have the four stations at 6,000 m as additional reference stations are not needed There are several stations in the current grid that can be used for comparison/reference purposes including the two outermost stations along radial 1 and 5 (Figure 3-2). Note that this design is based on the current HWT design plan and anchor patter. However, once all HWT-related infrastructure is installed, it is highly probable that some stations may have to be relocated to ensure adequate asset protection. Similar to HSE, the actual or as-built anchor pattern will be used to determine if the baseline stations can be retained or relocated.

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Appendix A lists the updated coordinates for the Hibernia GBS, HSE, and HWT sediment sampling program. After each EEM survey, the sampling grid will be re-examined to determine its effectiveness and whether modifications are necessary.

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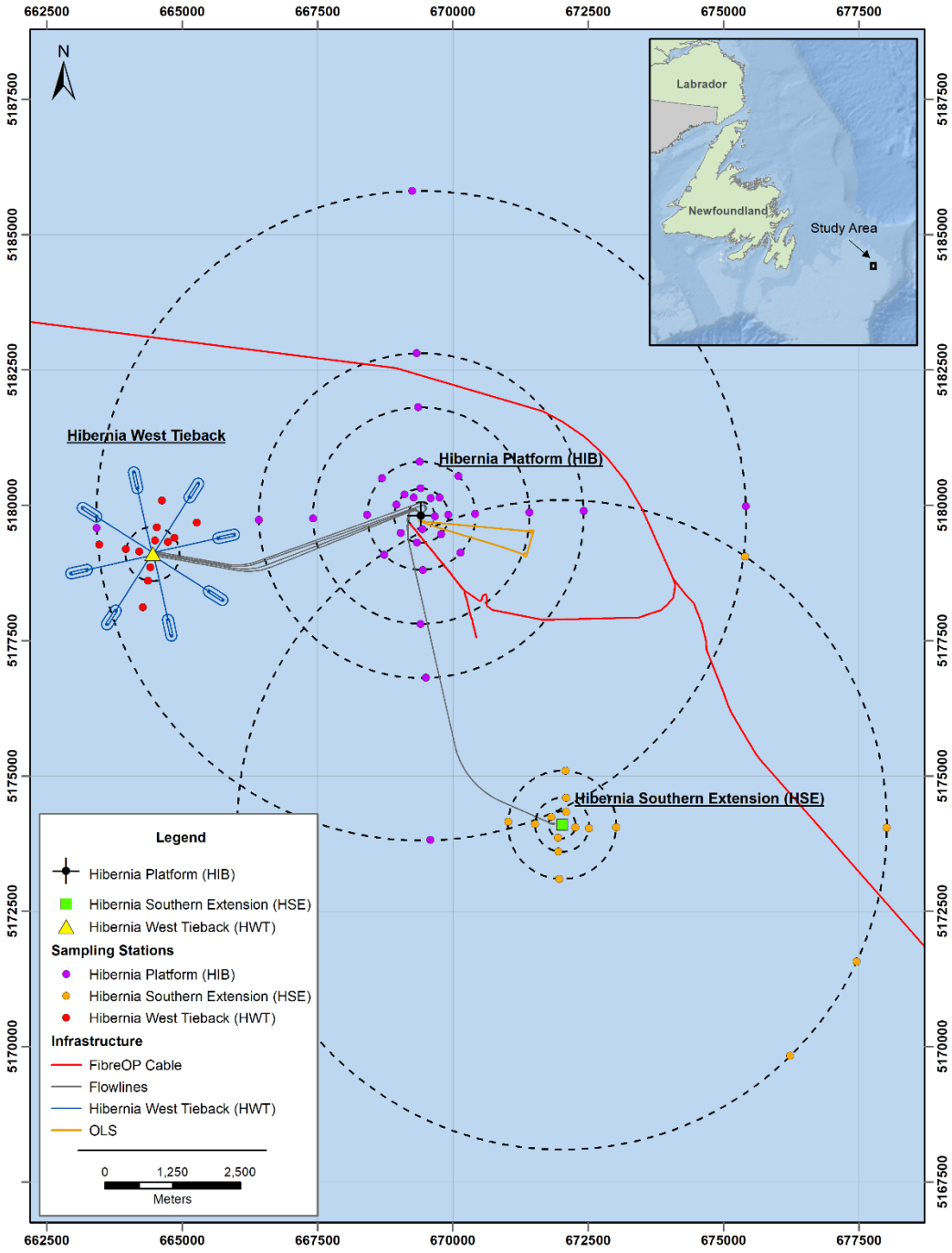

Figure 3-2: Sediment Sampling Grid for Hibernia Platform, HSE, and HWT

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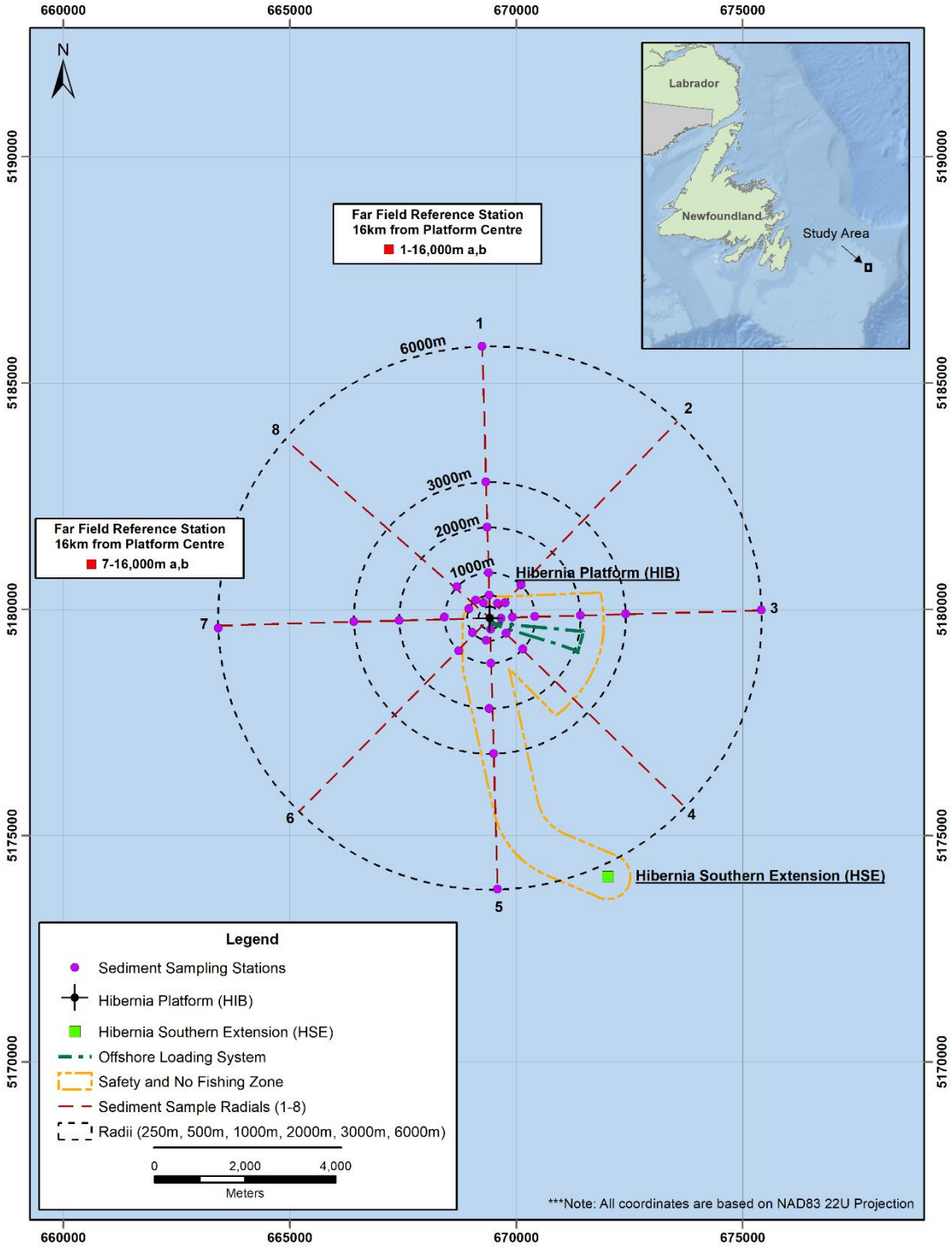

Figure 3-3: Sediment Sampling Grid for Hibernia Platform

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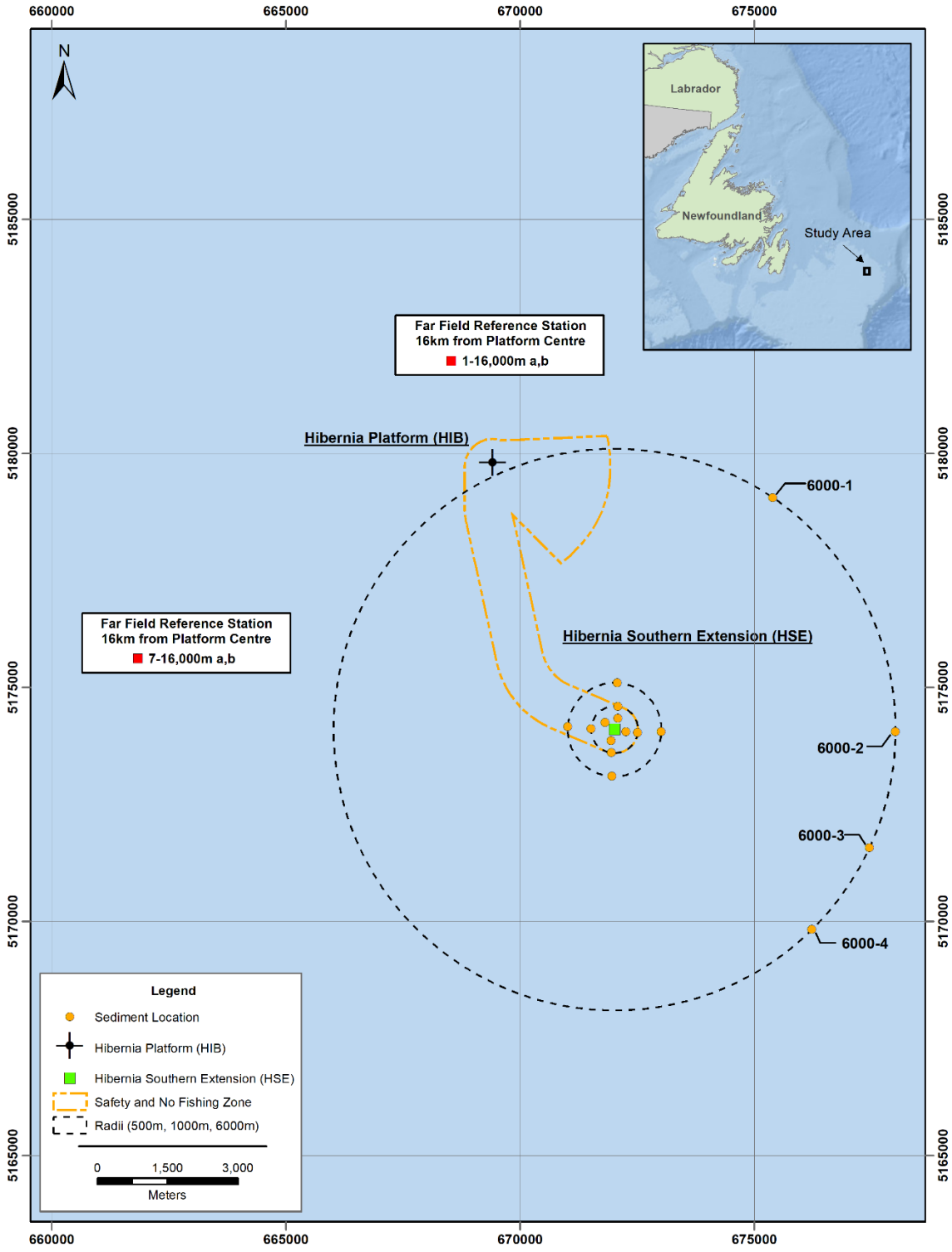

Figure 3-4: Sediment Sampling Grid for HSE

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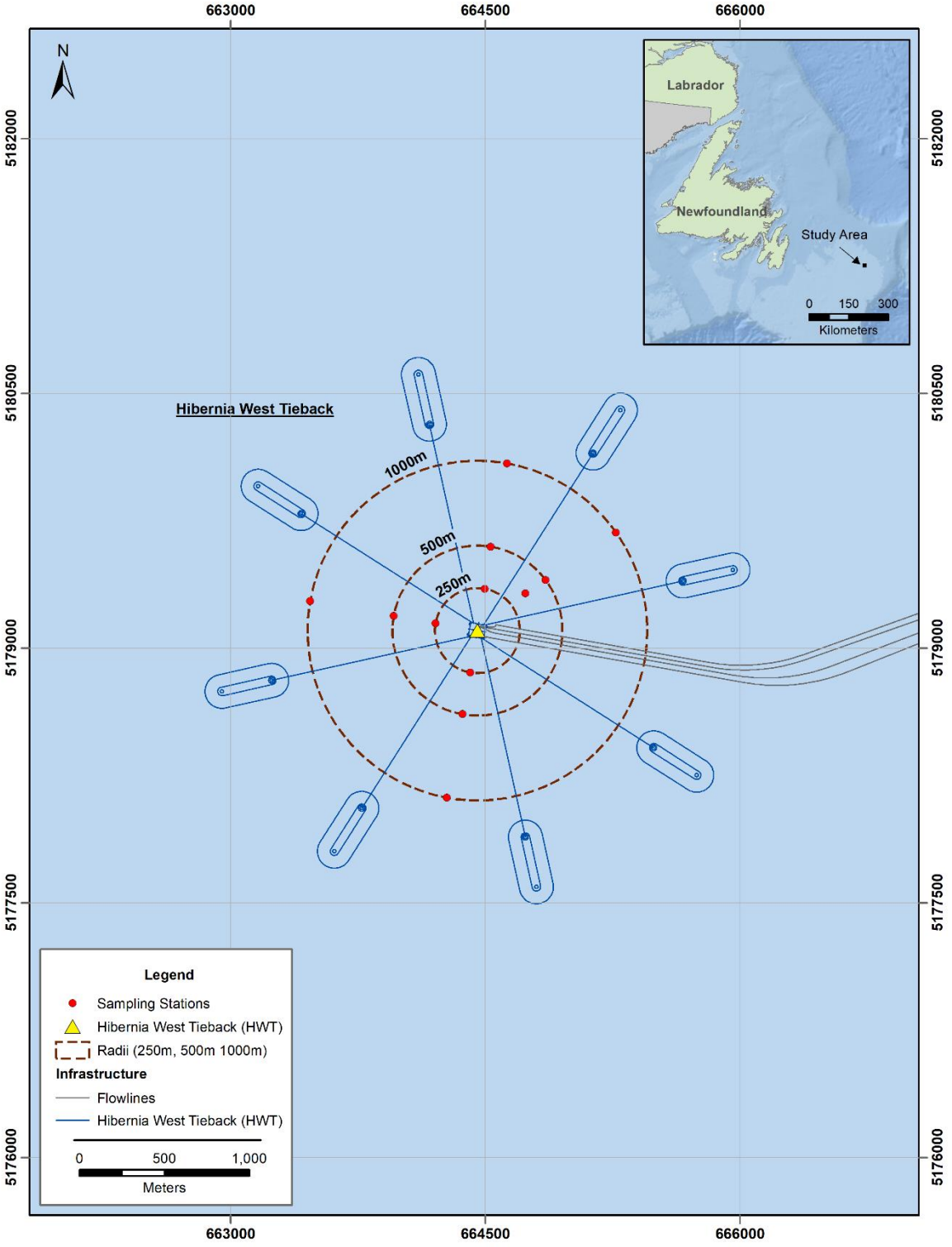

Figure 3-5: Sediment Sampling Grid for HWT

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3.3 Sediment Sampling Frequency

Similar to the previous versions of the plan, the sediment and commercial fish components of the EEM program for Hibernia and HSE will implemented every other year with the next field program currently planned for 2020. For HWT, baseline is scheduled for the Summer of 2019 for Sediment Sampling and 2020 for Commercial Fish Sampling. The Operational EEM for HWT will commence in 2023 after drilling is commenced (scheduled for late 2022) and will occur every year for the first three years. After this, and based on results, the implementation frequency may decrease.

3.4 Sediment Collection Methods

3.4.1 Sediment Collection Method (GPS and Box Corer)

As with previous EEM programs, a differential global positioning system will be used to ensure the accuracy of station positions. During previous sampling campaigns. Positioning has been typically provided by StarPack DGNSS with differential corrections.

Each sediment sample will be collected using a modified Pouliot box corer with a stainless steel core barrel capable of sampling an area of 0.10m^2 of the sea floor to a maximum depth of 60cm (Figure 3-6). Samples for sediment chemistry will be collected from an undisturbed 0-5 cm layer of the core. Sediment toxicity samples will be collected from an undisturbed 0- 7.5 cm layer of the core.

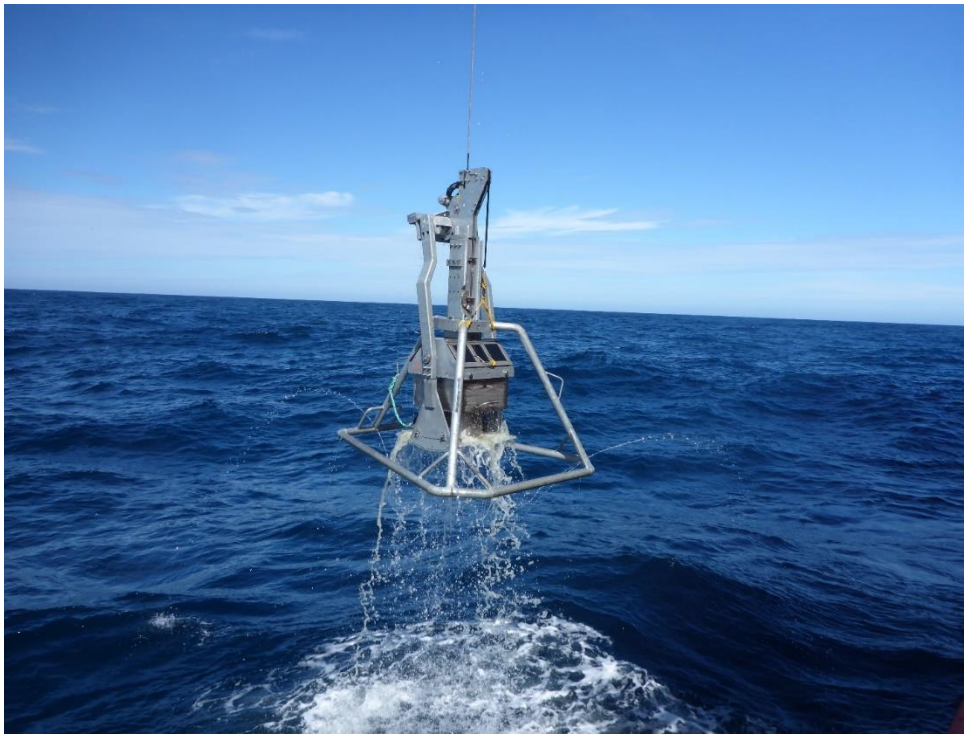

Figure 3-6: Modified Pouliot Box Corer

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A single grab sample will be collected at each sediment sampling station. For QA/QC purposes, a total of 10% of all stations will be sampled in duplicate. Figure 3-7 outlines the sediment sampling strategy for the EEM program. Before any of the sampling jars are filled, the thickness of the core will be measured, the redox potential and temperature measured, and a photograph of the core taken. Included in the photograph will be a placard which identifies the sampling station at which the core was collected. From each core, samples will be collected for metals, total petroleum hydrocarbons, BTEX, polycyclic aromatic hydrocarbons, sulphide, total inorganic carbon, total organic carbon, total carbon, particle size analysis and an archive sediment sample. Sediment samples collected for chemical analysis will be placed into onboard freezers maintained at -20°C. The collection of samples for chemical analysis will require the use of approximately half of the sediment core, with the remaining half used for toxicity analysis. This entails removing the 0-7.5 cm surface layer using clean stainless steel tools and placing it into a food grade plastic bucket lined with a polyethylene bag. Once the bucket is full, it will be sealed with a gasketed lid. Toxicity samples will be maintained at 4°C. The temperatures of all fridges and freezers will be regularly monitored (a minimum of every three hours) and the temperature recorded. All stations will be subject to the same sub-sampling strategy.

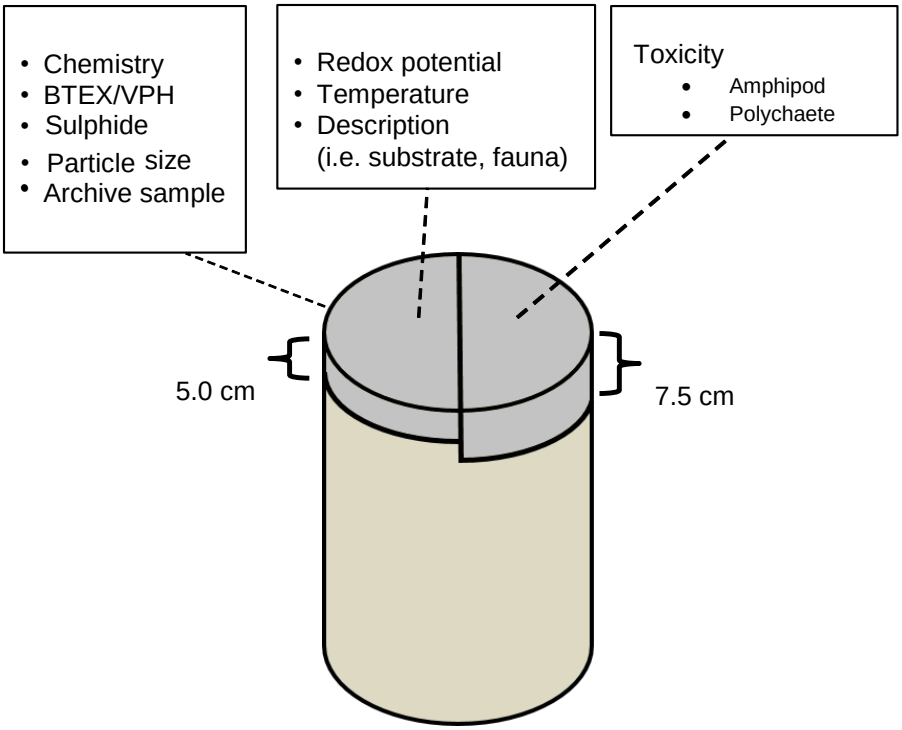

Figure 3-7 Hibernia Sediment Sampling Strategy

3.4.2 Sediment Chemistry Methods

After collection, samples will be held at -20°C until delivered to the respective lab for analysis. Where applicable, analytical laboratories will be accredited to ISO/IEC 17025:2005 by a recognized accrediting body, such as the Standards Council of Canada or the Canadian

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Association for Laboratory Accreditation. The detection levels will be as low as practically possible. Detailed analysis methodologies are contained in Appendix B.

3.4.3 Sediment Toxicity Methods

The sediment toxicity assays are to be conducted according to standard methods established by Environment Canada and Puget Sound Estuary Program (PSEP 1991,1995). Methodology details for bioassays are provided in Appendix B.

Sediment needed for toxicity testing will be sampled as per sediment chemistry field collection methodology (Section 3.1.3). One sample per station. All collected samples will be initially screened via the Petrotox guideline for sediment toxicity (i.e., C10-C21 hydrocarbons >150 mg/L), commencing immediately upon receipt of the samples by a SCC ISO17025-accredited laboratory**.** Any samples that are above the Petrotox guideline will then be tested for Amphipod Survival and Juvenile polychaete. All Reference Stations outside the influence of discharges will be subjected to the full suite of sediment toxicity assays. All toxicity testing will be conducted within six weeks of sample collection as per Environment Canada guidelines.

The sediment toxicity assays to be used during production phase EEM surveys include Juvenile Polychaete and Amphipod Survival assay. Sediment toxicity test details are presented in Table 3-7. Lethal endpoints measure survival over a defined exposure period. The amphipod and polychaete tests use whole sediment. The Petrotox test measures the potential for lethal and sub-lethal effects, whereas the amphipod survival tests measure lethal effects.

Table 3-7: Toxicity Test Particulars

Toxicity Test	Amphipod Survival	Juvenile Polychaete
Endpoints: Biological	% survival	% survival and % difference in growth
Endpoints: Statistical	Significant difference from negative control or reference	Significant difference from negative control or reference
Reference Method	EPS 1/RM/26	PSEP 1995
Test Organism	*Rhepoxynius abronius*	*Polydora cornuta,* *Neanthes arenaceodentata*
Test Initiation	Within 6 weeks of collection	Within 6 weeks of collection
Test Termination	10 days after test initiation	20 days after test initiation
Performance Criteria	90% average survival in negative control sediment	90% average survival in negative control sediment

Petrotox

Application of this Petrotox sediment quality guideline will enable identification of sediments posing little or no risk to the environment and therefore will not be subject to further statistical and

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laboratory analysis. The no effects concentration level obtained via applivcation of the PetroTox tool was 150 mg/kg, with a range of 115 to 190 mg/kg (Stantec 2013).

Amphipod Survival Assay

The Amphipod Survival Assay is conducted following EPS 1/RM/26 and EPS 1/RM/35 procedures (Environment Canada, 1992, 1998) using the marine amphipod *Rhepoxynius abronius*. The test procedure requires that five one litre replicate test chambers be established for each sample. Within each test chamber, 2 cm of sediment is placed in the bottom, 800 ml of water is added and 20 test organisms. Additional test chambers for negative control, positive control and water quality are also run simultaneously. The test is allowed to run for 10 days under prescribed test conditions at which point survival of the test organisms is determined. Survival in test sediments is compared to the result of the negative control to determine sediment final toxicity.

The sediment toxicity assays are to be conducted according to standard methods established by Environment Canada and Puget Sound Estuary Program (PSEP 1991,1995). Methodology descriptions for bioassays are provided in Appendix B.

Juvenile Polychaete Growth Assay

The statistical endpoint for the juvenile polychaete toxicity test is the determination of whether the biological endpoint (percent survival and mean growth) differs statistically from the control or reference sample, calculated using TOXCALC computer program (Tidepool Scientific Software 1994). Environment Canada has no interpretative guideline for the juvenile polychaete toxicity test. For the purpose of this report and as an aid for the interpretation of the results, the amphipod survival tests interpretative guidelines will be used as a guide for the interpretation of the juvenile polychaete data. Therefore, the juvenile polychaete survival test results would be considered toxic if the endpoint (mortality) is more than 30 percent lower and significantly different than mortality in the negative control sediment at the p=0.05 level. The juvenile polychaete growth test results would be considered toxic if the endpoint (mean growth) is more than 30 percent lower and significantly different than mean growth in the negative control sediment at the p=0.05 level.

Alternatively, the juvenile polychaete survival test results would be considered toxic if the endpoint (mortality) is more than 20 percent lower and significantly different than mortality in Reference site sediment at the p=0.05 level. The juvenile polychaete growth test results would be considered toxic if the endpoint (mean growth) is more than 20 percent lower and significantly different than mean growth in the Reference site sediment at the p=0.05 level.

3.4.4 Sediment Program QA/QC

The field staff and members of the vessel crew involved with sampling will be advised of the sampling plan and sampling procedures prior to the commencement of the sediment sampling program. Work instructions and standard operating procedures which outline the procedures to be used when sampling (sample collection and handling) will be available at all times and will be maintained in a project specific sampling plan. Collected data will be recorded on standardized data sheets and samples will be collected in laboratory supplied pre-labeled sample bottles.

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The analytical lab will follow standard quality control practices. These QC practices include following standard accredited reference methods for analyses and running laboratory duplicate samples to ensure that results are consistently within acceptable tolerances. Prior to the use of results for EEM purposes, laboratories will complete data validation and verification checks.

Sediment samples will be collected within 50m of a pre-determined positions. A sample from each station will be archived for 12 months in the event additional replicate analyses are requested. Duplicate samples will be also be collected at randomly selected locations for Hibernia, HSE, and HWT stations (10 percent of total number of stations) and will be subject to the same sub-sampling strategy as other stations.

The results of analytical analyses will undergo a quality review. This review will include screening for outliers and abnormal results. Any abnormal data will trigger a review of the raw data to ensure that the reported result was not as a result of human error (e.g. transcription error) by either the environmental firm carrying out the work or the analytical lab. If abnormal results remain after the data review, the archive sample can be sent for confirmatory analysis.

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4.0 WATER QUALITY

The overall purpose of the water column program are:

- to validate predictions generated dispersion models,
- to monitor contaminants associated with produced water releases and their dispersion in the water column; and
- to predict a zone of potential effects based on available chemical and toxicity data.

Water Quality sampling will only occur at and around the Hibernia platform. The HSE and HWT EDC locations will have no water quality component as they will not be subject to produced water or any other process related liquid discharges.

Results from past produced water sampling (e.g., HMDC 2018) indicate that the produced water plume dilutes rapidly once it is discharged, as analytes detected in seawater in the vicinity of the Hibernia platform are not typically detected beyond 50 m from the produced water discharge point. Moreover, the most volatile and abundant hydrocarbons in produced water from the Hibernia platform have been BTEX (HMDC 2018, Yeung et al. 2015). BTEX analytes have estimated concentrations not exceeding 0.37% of undiluted produced water concentrations approximately 31 m from the produced water discharge point, indicating that their potential for affecting a deleterious biological effect is likely minimal. Consistent with this, in a recent study characterizing the physical, chemical and microbial properties of produced water and seawater from the Hibernia platform, all analyses revealed that the discharge of produced water did not have a detectable effect on the surrounding seawater (Yeung et al., 2015).

Based on review of results, two changes have been incorporated into the Water Quality portion of the EEM Program:

- Limit sampling to no closer than 50 m from the GBS (Section 4.3).

- CTD no longer required to identify produced water plume location to direct sampling inside 50m (Sections 4.3 and 4.6.1).

Further explanation and rationale for this change is provided in the referenced section.

4.1 History

Water column chemistry monitoring associated with produced water was initially incorporated as a pilot program in conjunction with the 2004 Hibernia EEM program and is now included as a fixed monitoring component in the core EEM program. The water quality component has had several iterations over the years as detailed below.

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4.1.1 2004 Pilot Program

A water quality pilot study was undertaken during the 2004 EEM program to assess the effectiveness of using conductivity-temperature-depth (CTD) profiles to identify the location of the produced water plume and to use water chemistry results to validate the predictions of the Hibernia produced water dispersion model (Lorax Environmental 2003). The chemical analyses focused on TPH, PAH and select metals. Information contained within the Hibernia EIS (Mobil 1985) indicated worst case dilutions of 1:1,000 would be achieved within 1 km and dilutions of 1:10,000 within 8 km. Dilution ratios as predicted by the Hibernia produced water model (Lorax Environmental 2003) are 1:100 to 1:3,000 within 50 m from the discharge point, depending upon flow rates. Dilutions of 1:10,000 are predicted to be achieved beyond 750 m regardless of flow rates.

The objectives of the pilot program were to:

- assess the effectiveness of using CTD profiles to identify the produced water plume; and
- use water chemistry results to validate the predictions of the Hibernia produced water dispersion model which predicted a much smaller zone of influence than within the EIS.

To ensure water column samples collected at Hibernia were reflective of the influence of the produced water plume, the location of the plume was identified in “real time” using differences in temperature, conductivity, salinity and dissolved oxygen, as measured with a CTD profiler. CTD profiles of the water column within 50 m of the produced water outlet clearly differed from profiles conducted at the Reference sites.

The pilot study initiated sampling as close to the Hibernia produced water outlet as possible. The actual sample locations were weather-dependant and located at a distance deemed safe by the vessel master. Stations radiated out from the initial station as far as the plume could be tracked as indicated by the results of the CTD profiles. This was anticipated to be within 500 m of the Platform but was determined to be within 40 m of the Platform. Sample locations as directed by the CTD scan results (2004) are illustrated in Figure 4-1.

Once the plume was identified, 10 L Niskin samplers were deployed to obtain water samples at two locations within the plume and at locations near-surface and near-bottom. The use of CTD profiles successfully directed sample collection from within the produced water plume for 10 of 11 stations. All samples were analyzed for fuel and lube range hydrocarbons, BTEX, PAH and metals. The results of water analysis were then used to validate the predictions of the produced water dispersion model (Lorax 2003).

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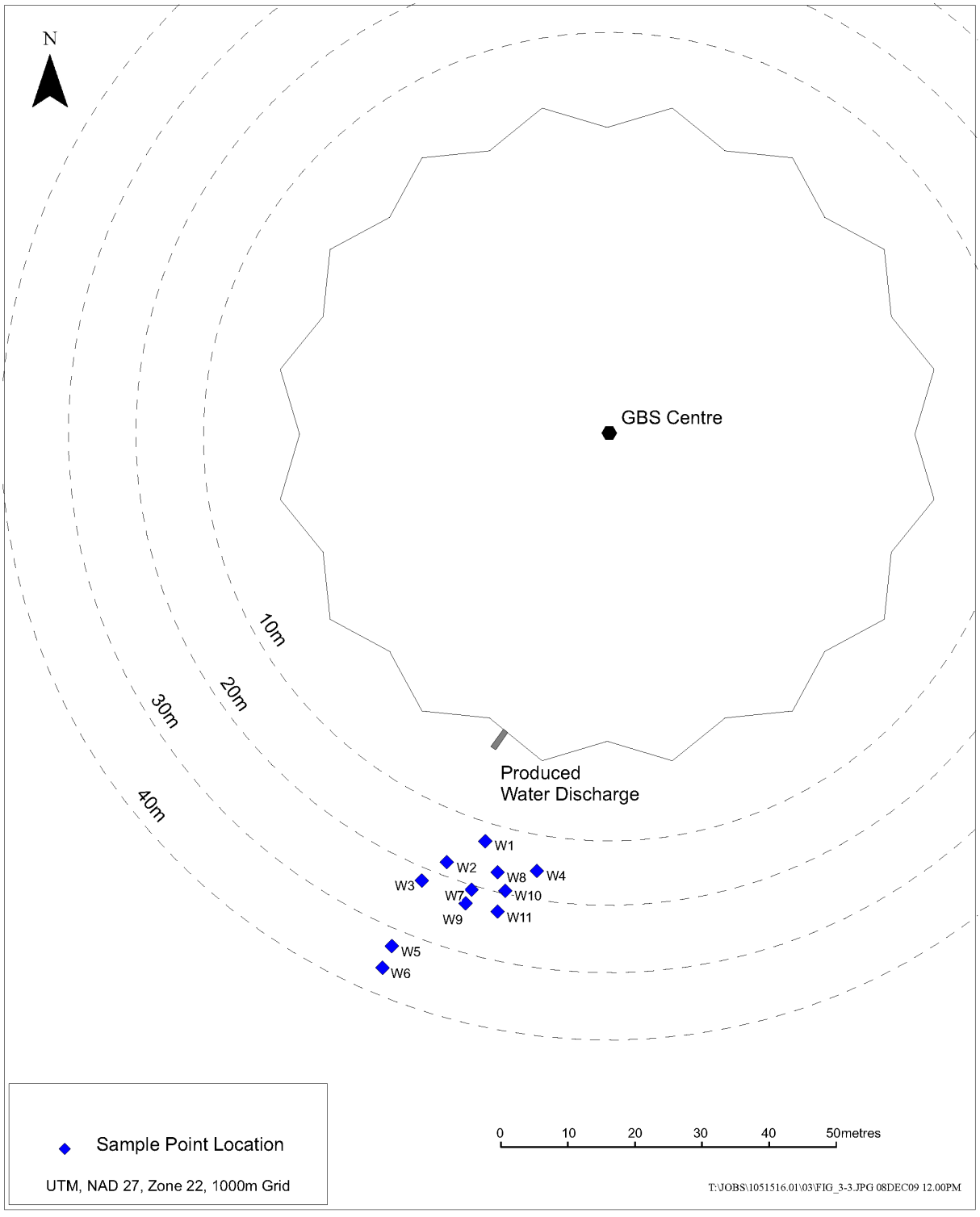

Figure 4-1 Water Quality Station Locations Hibernia 2004 EEM Program within delineated discharge plume

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4.1.2 2007 Amended Program (2007-2018)

The sampling design for the 2007 water column chemistry program was revised based on a review of data collected in 2004. The objectives of the 2007 program were as follows:

- to determine estimates of dispersion factors to validate dispersion modelling; and
- to predict a zone of potential effects from available chemical and toxicity data.

In 2007, as in 2004, CTD profiles were used to locate the produced water plume. CTD profiles conducted within 50 m of the discharge (near-field) clearly differed from the Reference sites (far-field) profiles, while profiles conducted at 50, 100 and 200 m (mid-field) were practically indistinguishable from Reference Site profiles. Water sampling locations for the 2007 EEM are illustrated in Figure 4-2. Representative CTD profiles for the near-field, mid-field and far-field Reference sites are presented in Figure 4-3.

A series of 10 L Niskin water samplers were used to obtain two samples within the produced water plume, one at the surface and a near-bottom at each sample station. Samples were analyzed for the same parameters as 2004 with the addition of nutrients (total nitrogen, ammonia nitrogen and total phosphorous). Sampling of “raw” produced water on the Hibernia Platform was conducted concurrently with the 2007 water column sampling to obtain undiluted concentrations and constituents.

Dilution factors were calculated by comparing field concentrations of target parameters with concentrations in “raw” produced water on the Hibernia Platform. Dilution factors ranged from 94 (at the 27 m station) to greater than 13,505 (at the 22 m station). The calculated concentrations of produced water ranged from 0.004 to 1.06 percent at the 22 and 27 m stations, respectively. The field-derived produced water concentrations correspond well with the produced water concentrations of 0.06 to 0.7 percent predicted by the produced water dispersion model (Lorax 2003).

When field-derived concentrations of produced water were compared to laboratory produced water toxicity data (generated to satisfy Offshore Waste Treatment Guidelines (NEB et al. 2010) requirements, it was shown that concentrations required to achieve toxicity do not extend beyond 22 to 27 m of the Hibernia produced water discharge. Thus, a zone of potential effects can be generally stated to exist within 50 m of discharge outlets. This concurs with dispersion modelling studies conducted world-wide that all predict a rapid dilution in the range of 30- to 100-fold within the first tens of metres of the discharge point (Terrens and Tait 1993; Brandsma and Smith 1996; Neff 2002). Therefore, the model predictions in the EIS with respect to produced water are very conservative and will most likely be achieved much closer to the discharge points than those noted in the EIS (Mobil 1985).

The results of water analysis from 2007-2018 have validated the predictions of the produced water dispersion model (Lorax 2003) and the EIS (Mobile 1985) that the zone of potential effects is within 50 m of the discharge outlets. Therefore, stations within 50m of the GBS can be eliminated but the more distal locations will continue to be monitored to verify dispersion model predictions

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and our understanding of the limited extent of a potential zone of effects arising from the release of treated produced water.

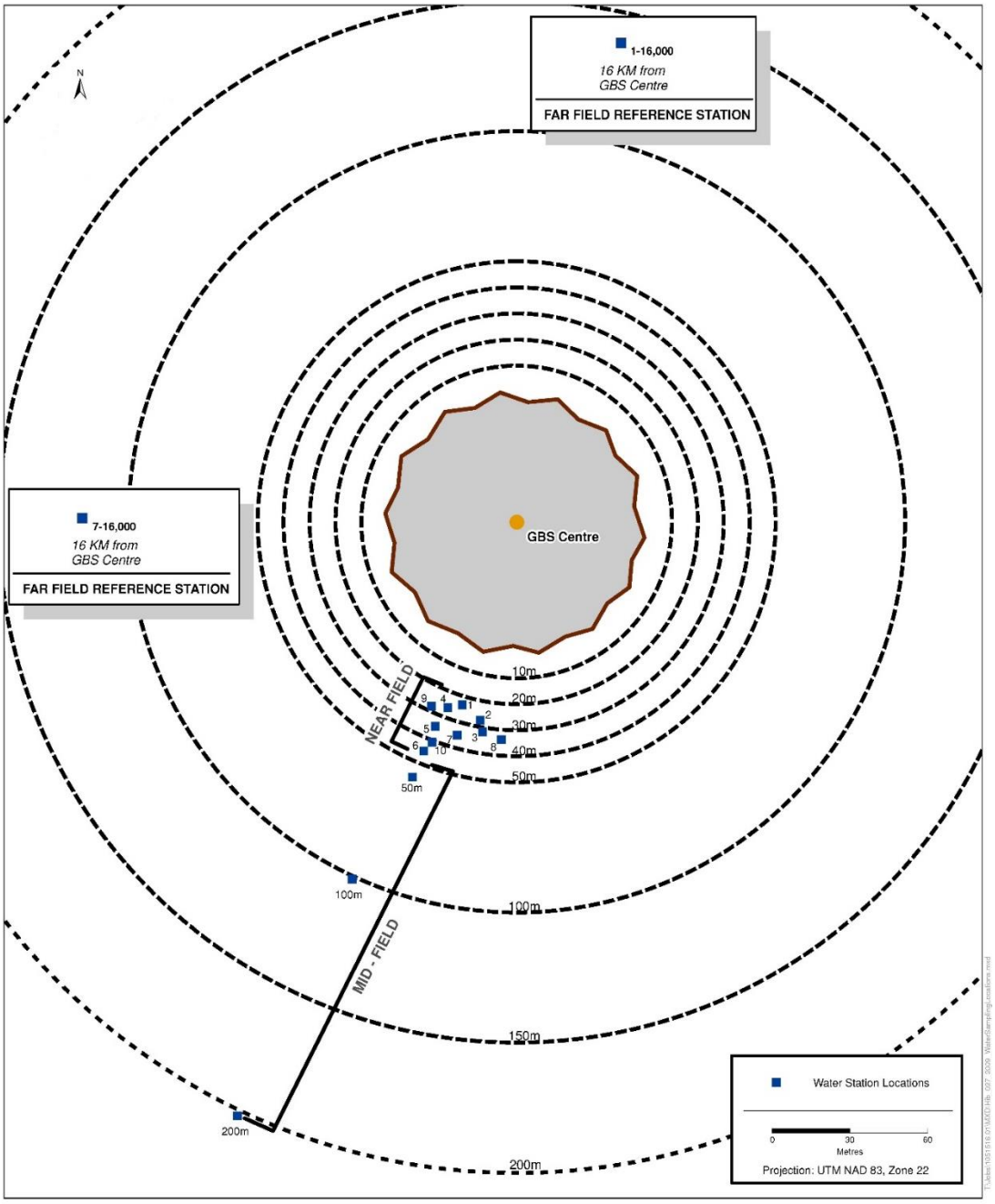

Figure 4-2 Water Sampling Locations 2009-2018 EEM (note nearfield <50m stations have varied)

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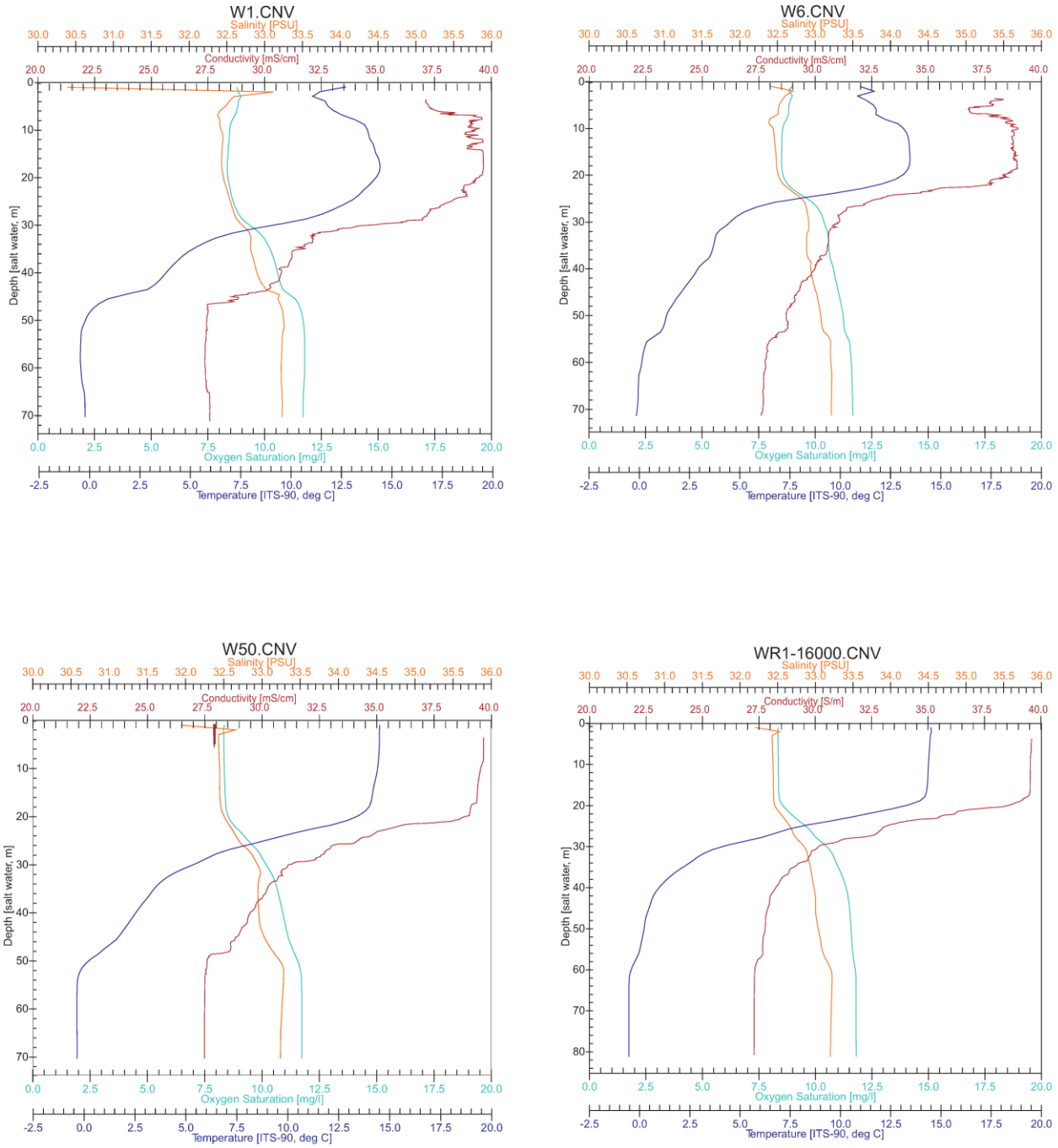

Figure 4-3 Graphic Display of Representative CTD Profiles at Near-field (W1 and W6), Mid-field (W50) and Far-field (WR1 – 16,000) Sampling Stations

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4.2 Water Quality Parameters

The parameters analysed for the water quality sampling are listed in Table 4-1. These are the same analytes that were measured in previous years and included in the last iteration of the EEM design (HMDC 2013). Unlike sediment quality and commercial fish sampling, alkyl PAHs have been retained for water quality as they have been detected previously in the water column (HMDC 2018).

Table 4-1: Water Quality Parameters to be Analyzed during EEM Programs

Metals	Other	PAHs	
Arsenic	Total Suspended Solids (TSS)	1-Chloronaphthalene	Fluorene
Barium	Oil and Grease	1-Methylnaphthalene	Indeno(1,2,3-cd)pyrene
Cadmium	Chlorophyll *a*	2-Chloronaphthalene	Naphthalene
Chromium	Phaeophytin *a*	2-Methylnaphthalene	Perylene
Cobalt	**Hydrocarbons**	Acenaphthene	Phenanthrene
Copper	BTEX	Acenaphthylene	Pyrene
Iron	C6 to C10 (less BTEX)	Anthracene	C1 – Dibenzothiophenes
Lead	>C10 to C21	Benzo(a)anthracene	C2 – Dibenzothiophenes
Manganese	>C21-C32	Benzo(a)pyrene	C3 – Dibenzothiophenes
Nickel	**Nutrients**	Benzo(b)fluoranthene	C1 - Phenanthrenes
Strontium	Ammonia	Benzo(g,h,i)perylene	C2 - Phenanthrenes
Zinc	Nitrogen	Benzo(k)fluoranthene	C3 - Phenanthrenes
Mercury	Phosphorous	Benzo(e)pyrene	C1 - Naphthalenes
	Total Organic Carbon	Chrysene	C2 - Naphthalenes
Radionulciotides (Radium 226, 228 and Lead 210)		Dibenz(a,h)anthracene	C3 - Naphthalenes
		Fluoranthene	Dibenzothiophenes

4.3 Water Quality Stations

The Water Quality Station Design is largely consistent with previous EEM Plan Designs (HMDC 2013) with the exception that the real-time stations located within 50 m of the platform will be removed. Water column sampling previously consisted of fixed and real-time stations (Figure 4-4). The rationale for removing the real-time stations are as follows:

- The original objective of the sampling <50 m has been met; the produced water plume located has been verified as well model predictions and limited extent of potential zone of effects (See 4.1.2)

- Results from the Risk Assessment (Section 1.4.3) determined that there was a risk of the vessel making contact with south lifeboat station and/or the GBS (See 1.4.3)

Water quality monitoring at the more distal locations will continue to be monitored to verify dispersion model predictions and our understanding of the limited extent of a potential zone of effects arising from the release of treated produced water. Figure 4-4 illustrates the proposed water sampling plan going forward.

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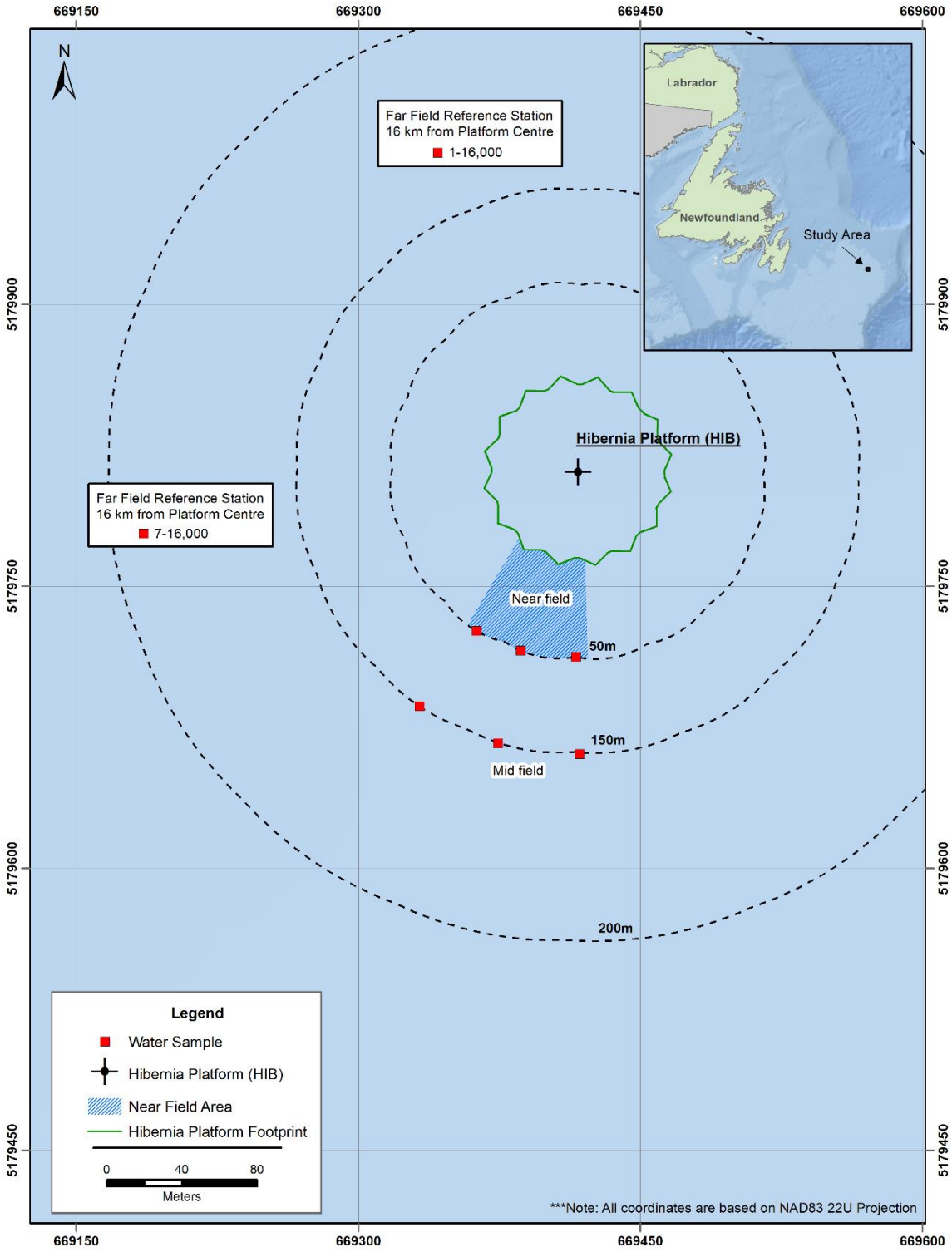

Figure 4-4: Planned Water Sampling Stations

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Water column locations sampled during at 50 m (3 stations) and 150 m (3 stations) will remain fixed stations going forward.

4.4 Water Quality Frequency

Water quality sampling occurs on the same cruise used for sediment sampling with the same schedule. No water quality sampling is planned for HSE or HWT due to the absence of process related liquids discharges such as produced water.

4.5 Effluent Characterization (Produced Water)

If logistically feasible, Offshore Waste Treatment Guidelines (NEB et al. 2010) sampling (including toxicity testing) will be conducted on the Hibernia Platform concurrent with field sampling associated with the water column program. Similar to Water Quality sampling, Effluent Characterization samples will be analyzed for fuel and lube range hydrocarbons, BTEX, PAH, metals, nutrients, oil and grease, total suspended solids, chlorophyll *a* and phaeophytin *a* (refer to Table 4-1 for a list of metals and PAHs to be analyzed).

4.6 Water Column Program Field Plan and Analysis

4.6.1 Water Sampling Methods

Similar to sediment sample locations, DGPS will be used to ensure accuracy of station positioning. At a single station, CTD profiles will be conducted to give context to the water chemistry results (Figure 4-5). 10 L Niskin water samplers will be deployed to obtain three water samples (one near-surface, one mid-surface and one near-bottom) at each sampling station. A minimum of eight locations will be sampled including near-field (3), mid-field (3) and reference stations (2). Additionally, 10 percent of stations will be sampled in duplicate (at one depth, surface, mid or bottom) for QA/QC purposes. Standardized data sheets will be completed for each sampling station.

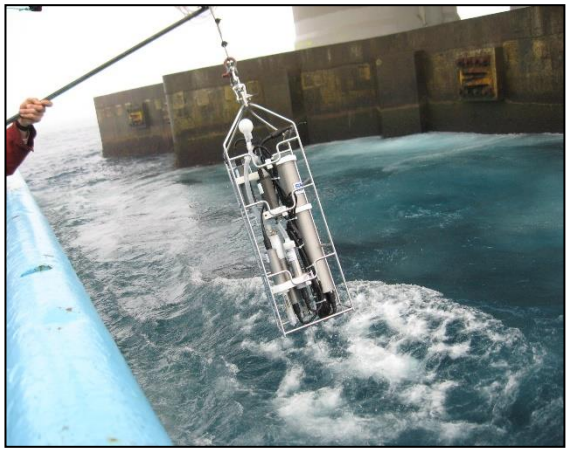

Figure 4-5: Deploying CTD

Water Quality Samples will be decanted from the Niskin water sampler into pre-labelled, laboratory-supplied sample bottles. All samples will be kept refrigerated at or near 4°C in a dedicated freezer. The temperature of the freezer will be monitored and recorded every three hours, until delivery to a certified analytical laboratory.

Chlorophyll and phaeophytin samples are collected from the Niskin Samplers in 1 L plastic bottles that are wrapped in tin foil to protect the sample from direct sunlight (to avoid degradation of chlorophyll pigments). Samples are filtered within 30 minutes of collection by use of a vacuum filtration apparatus. Each 1 L sample is gently filtered (less than 75 mm Hg vacuum pressure) onto a GF/F filter and the sample is “fixed” by adding approximately 20 ml of magnesium

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carbonate solution. After filtration, each filter pad is wrapped in tin foil, placed in a pre-labeled petri dish, taped and stored at -20 ºC.

4.6.2 Sample Analysis Methods

Analytical methods for water analysis are contained in Appendix C.

4.6.3 Analysis

Based on the measured concentration of these parameters, some will be used in conjunction with raw produced water data to provided estimates of dispersion factors. The dispersion factors will then be used to predict potential zones of influence associated with raw produced water data.

Dilution factors for key parameters will be calculated by comparing field concentrations of select parameters with concentrations in “raw” produced water on the Hibernia Platform (Effluent Characterisation). To estimate the dispersion of analytes in produced water relative to the concentrations detected from samples collected in the water column surrounding the Hibernia Platform, dispersion and percent concentration will be calculated as follows:

$$\%\ Dispersion = \frac{Produced\ water\ concentration}{Analyte\ concentration\ in\ field}$$

$$\%\ Concentration = \frac{Analyte\ concentration}{Discharge\ concentration}$$

4.6.4 Quality Assurance

A sampling plan will be prepared and distributed to all field staff and relevant members of the ship’s crew prior to commencing the water sampling program. Standard operating procedures outlining methods for sample collection and handling will be included in the sampling plan. The CTD profiler will be calibrated and documented by the supplier prior to use. All data will be recorded on standardized data sheets and samples will be collected in pre-labelled sample bottles.

The analytical laboratory will follow standard quality practices applicable to the analyses as required by the ISO17025 accreditation. The data will be verified and validated before provision of results for interpretation purposes (i.e., checked to ensure that all parameters have been analyzed and all results have been entered in the proper locations within the database). These QC practices include following standard accredited reference methods for analyses and running laboratory duplicate samples to ensure that results are consistently within acceptable tolerances. Prior to the use of results for EEM purposes, laboratories will complete data validation and verification checks.

Each sample collection position will be established using DGPS positioning and will be logged by personnel dedicated to this task. Ten percent of water quality stations will be sampled in duplicate for QA/QC purposes and will be subject to the same analysis as other samples.

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5.0 COMMERCIAL FISH

The purpose of the biological survey is to collect sufficient American plaice specimens for the assessment of tissue chemistry profiles, sensory evaluations (taint testing) and fish health indicators near the Hibernia production and drilling operations and at a Reference site approximately 50 km to the northwest.

Data on commercial fish species have been collected in each EEM monitoring year with fish health indicators included since 2002. The results of the fish health surveys and body burden analyses to date reveal that in general, there are no significant differences observed between fish collected from the Hibernia field compared to those collected from the reference area. Based on review of results, several changes have been incorporated into the Commercial Fish portion of the EEM Program going forward which include:

- Removal of alkly PAHs (see Section 5.1.1)
- Removal of archival sampling for organs (spleen, heart, kidney) (see Section 5.1.3)
- Increase in the size of the reference area (see Section 5.2)
- Change to the trawling locations (1000m separation to assets and debris) (see Section 5.2).

5.1 Commercial Fish Parameters

5.1.1 Tissue Chemical Profiles

Fish muscle tissue (dorsal fillet) and liver samples will be obtained from American plaice collected at the Hibernia field and at the Reference site as per earlier Hibernia EEM programs. Twelve samples for each tissue type will be collected from each site and analyzed for metals, TPH and PAHs. Method details are provided in Section 5.4.

A list of metal parameters to be examined in muscle and liver tissues for American plaice is provided in Table 5-1. This list of parameters remains the same as the minimum recommended metals from the original Hibernia EEM program design, with the exception of lithium. Lithium has been removed from the list of parameters previously (HMDC 2013) as it has been below detection limits for all of the Hibernia EEM programs to date. The hydrocarbons that will be analyzed are listed in Table 5-2. Note that alkylated PAHs and two other PAHs (biphenyl, dibenzothiophene) were added to the sampling design in 2009 but to date have not been detected are not part of the standard suite of PAHs and are therefore removed from the program.

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Table 5-1: Biota Trace Metal Parameters

Biota Trace Metal Parameters		
Arsenic	Copper	Mercury
Barium	Iron	Selenium
Cadmium	Lead	Zinc
Chromium	Manganese	

Table 5-2: Biota Petroleum Hydrocarbon Monitoring Parameters

Biota Hydrocarbons	
Total Petroleum Hydrocarbons (C6-C32) C6-C10 C10-C21 (Fuel Range) C21-C32 (Lube Range)	
Polycyclic Aromatic Hydrocarbons 1-Methylnaphthalene 2-Methylnaphthalene Acenaphthene Acenaphthylene Anthracene Benz(a)anthracene Benzo(a)pyrene Benzo(b)fluoranthene Benzo(g,h,i)perylene Benzo(j)fluoranthene	Benzo(k)fluoranthene Chrysene Dibenz(a,h)anthracene Fluoranthene Fluorene Indeno(1,2,3-cd)pyrene Naphthalene Perylene, Phenanthrene Pyrene
Alkylated and two other PAHs (biphenyl, dibenzothiophene) were sampled from 2009-2018 and will be removed from future sampling as they have not shown presence in the fish tissues.	

5.1.2 Sensory Evaluations (Taint Assessments)

Sensory evaluations or taint assessments will be conducted using the ventral fillets of American plaice. Evaluations will be conducted by taste panels using the triangle test; hedonic scaling assessment will be used only in the event of a positive triangle test result. Further details on the sensory evaluation testing and analysis can be found in Appendix D.

5.1.3 Fish Health Indicators

Fish health indicators will be examined from 60 fish collected from the Hibernia field and Reference site. Fish health indicators include the parameters and indices listed in Table 5 3. Additional detail on the fish health indicator analysis can be found in Appendix D. Similar to the rationale given in Section 3.1.1, only the spleen will be archived indefinitely and the other organs (heart, kidney) will no longer be retained indefinitely. Instead, the archives heart and kidney will be retained for 12 months from when they were initially taken or whenever raw data is released to the C-NLOPB.

Table 5-3: Fish Health Indicators

Fish Health Indicators	
Physical characteristics and Condition Indices	Megalocytic hepatosis

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Fish Health Indicators	
Total body weight	Eosinophilic foci
Gutted body weight	Basophilic foci
Length	Clear cell foci
Liver weight	Carcinoma
Gonad weight	Cholangioma
Age	Cholangiofibrosis
Condition index (gutted weight)	Increase in mitotic activity
Condition index (total weight)	Macrophage aggregation
Hepato-somatic index (HIS)	Hydropic vacuolation
Gonado-somatic index (GSI)	Parasite infection of biliary system
Mixed Function Oxygenase activity	Gill histopathology
Gross pathology	Thin lamellae
Haemotology	Distil hyperplasia
Lymphocytes	Epithelial lifting
Neutrophils	Clubbing with tip hyperplasia
Thrombocytes	Clubbing with telangiectasis
Liver histopathology	Basal hyperplasia
Nuclear pleomorphism	Fusion
	Oedema condition

5.2 Commercial Fish Sampling Locations

Previously, American plaice were collected near (>500 m, <2,000 m from the asset) the Hibernia Platform, the HSE, and at a Reference site 50 km northwest of Hibernia with similar depth and bottom characteristics. The fishing area was defined as > 500 m but less than 2,000 m from the asset or any infrastructure. The reference area was also defined as a 2,000 m radius.

Based on the April 30, 2019 risk assessment the 500m separation distance between otter trawl and subsea assets and debris is increased to 1000m (Section 1.4.3). This results in fish sampling occurring no closer than 1000m from subsea equipment or other hazards (debris). Due to the close proximity of subsea assets, this change results in the fish component assessing the effects at a Hibernia field level as opposed to assets within the field level as detailed below.

The no fishing area is shown in Figure 5-1. This area incorporates a 1000 m buffer around all subsea infrastructure including the Hibernia GBS, HSE, HWT, flowlines, anchor chains, fibre optics cable and other known hazards. As with HSE, HWT anchor chains will only be present while drilling. Once drilling is completed the fish trawl transects can be moved closer to the EDC. The fish trawl transects would take place just outside and adjacent to the 1000-m position. These data would be pooled together for the report and comparisons to the Reference Area in future EEM years. There will be a minimum of 10 trawls each for the Hibernia field and Reference Area.

This revised design is based on the following:

- Adoption of 1000m separation distance (safety buffer) between subsea assets and hazards and fish trawling;
- Review of other EEMs from other operators faced with similar situations with multiple drill centres; and

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- A review of previous commitments, statistical design, and hypotheses of the Commercial Fish Component to ensure that any revisions would still meet the original intent of the EEM Program.

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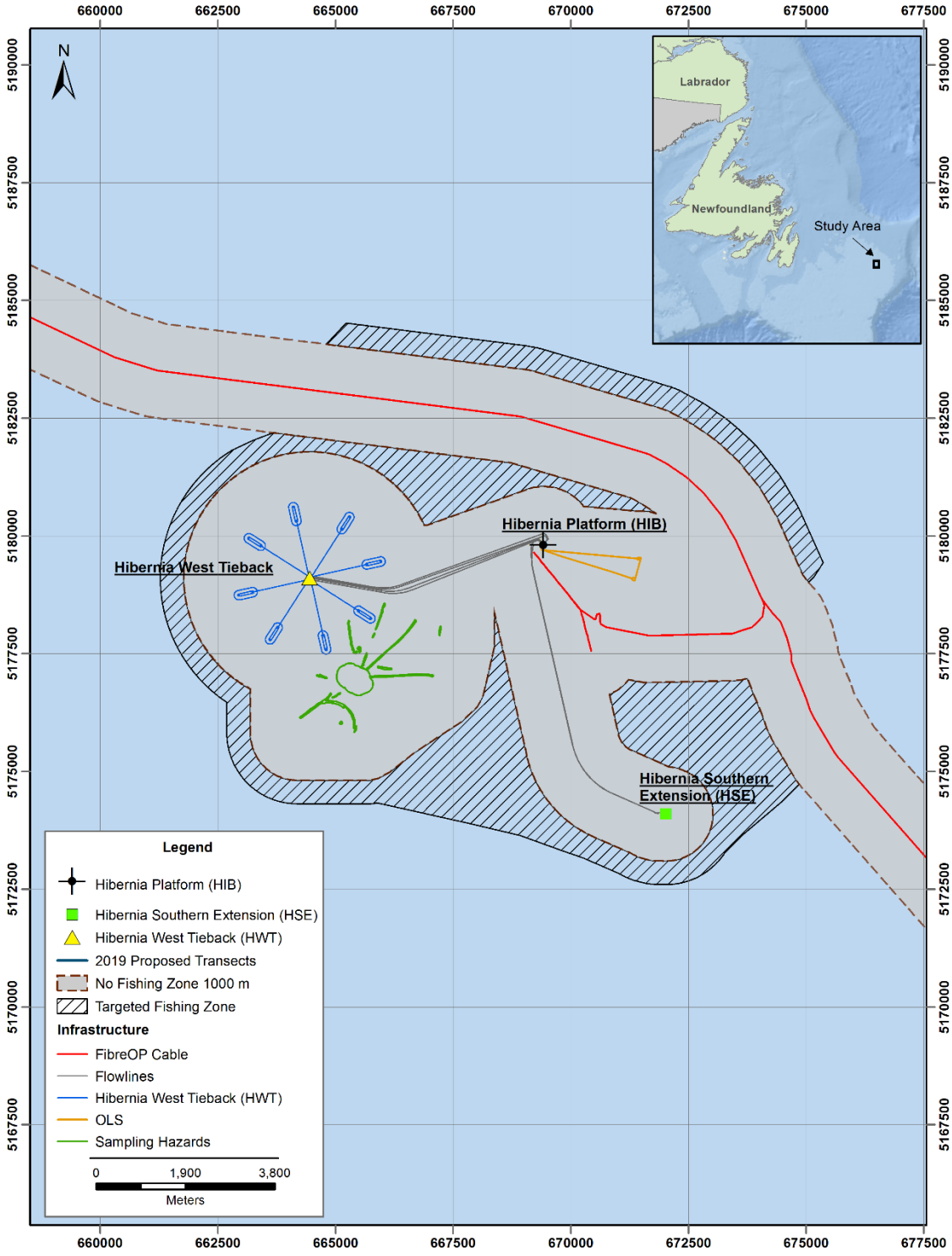

Figure 5-1 EEM Target Fishing Zones for Hibernia Field

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5.2.1 Risk Mitigation Strategy to Protect Subsea Infrastructure and Fishing Vessel

One of the findings from the risk assessment described in Section 1.4.3 was to increase the safety buffer between trawling and any infrastructure and debris to 1,000 m. During trawl towing, entanglement could occur with subsea infrastructure (OLS, SAL bases, flowlines, fibre optic lines, other debris, etc) resulting in a potential injury to fishing vessel personnel and /or equipment damage (vessel or subsea infrastructure). The mitigation identified for this hazard was to move the available trawling area (or 'fishing zone') to create a minimum separation distance of 1,000 m to adjacent infrastructure.

5.2.2 Comparison to Other Operators

The other operators with multiple drill centres (Terra Nova and White Rose) have essentially drawn a Fisheries Exclusion Zone that connects their drill centres and executed their commercial fishing EEM outside the exclusion zone polygon (Figure 5-2). Hibernia proposes to take a similar approach as discussed in more detail below.

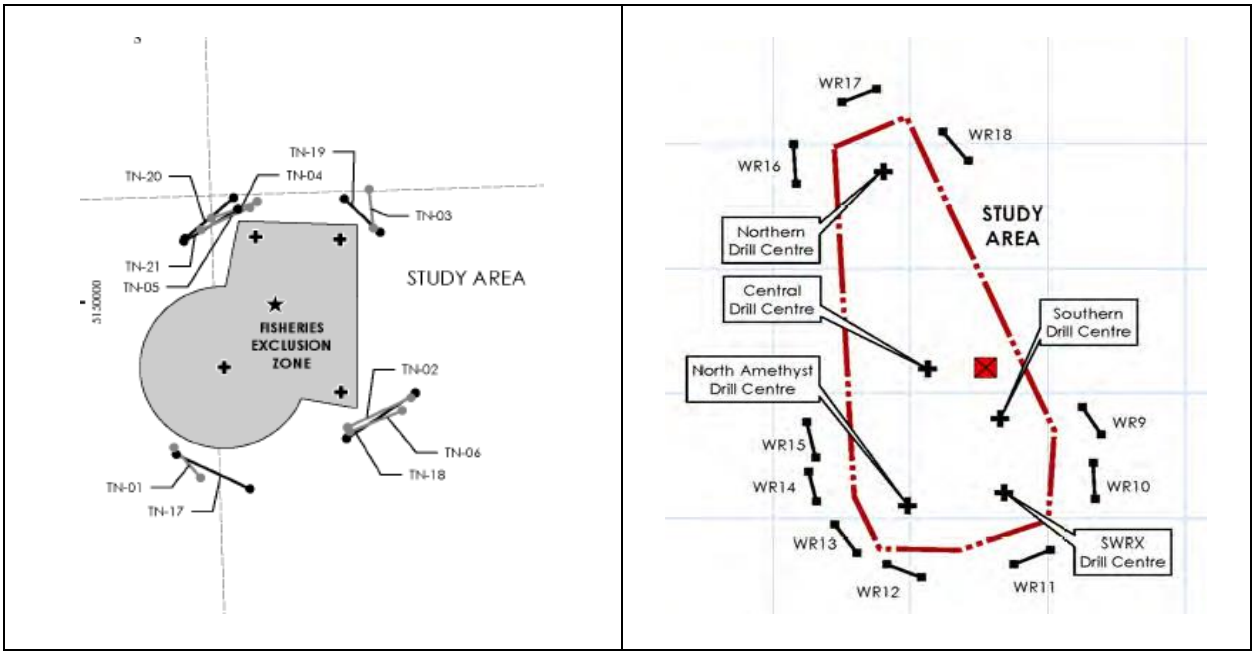

Figure 5-2 Fishing Transect for Terra Nova (left panel) and White Rose (right panel)

5.2.3 Assessment of Revising Commercial Fisheries Sampling Locations

One potential concern from a consolidated study area for the commercial fish program going forward is the potential loss of signal from any project effects resulting from the Hibernia Platform itself. The GBS has the potential to act as an artificial reef (Page et al. 1999, Sargent et al. 2006,

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Macreadie et al. 2011, Claisse et al. 2014), and past EEM years have found larger, older fish present at the Hibernia Platform (though this is not consistently detected in every year). The platform is the release point for produced water, the contact point for tankers, and the release of WBM and cuttings. Operation of the HSE drill centre will not result in process related discharges so potential effects are less likely than that from Hibernia Platform itself. Environmental risks at HWT will be similar to HSE after a short-term signal from installation and drilling. However, American plaice are a mobile fish species, and so it is possible fish capture at one location could be from a distant location, from another platform or from the Hibernia Platform area. The small distance between Hibernia and HSE and HWT is capable of being traversed by plaice. Summarized fish health program data from Hibernia and HSE is similar across EEM years (summaries for both are found in Table 5-4 and 5-5, respectively).

Currently, 10 samples for body burden are being taken for fish health at both Hibernia Platform and HSE. A combined sampling area for all three programs could increase the potential power of results. The weakest statistical points in the current program are the number of samples for taken for body burden (10). Going forward the body burden program will be improved by increasing the number of samples to 12. The amount of male fish caught has been an issue with past EEM years and other platforms, as they are smaller than females and fewer are typically caught (typical ratio at Hibernia is 2:5 males to females). Some platforms have simply excluded males altogether in certain years due to minimal or no males caught. A broader sampling area with a larger sample size may yield better results for the commercial fish program overall and would be consistent with the current EEM programs at other offshore productions facilities.

In this program going forward, there will be 12 samples collected for body burden from the Hibernia field overall with a minimum 10 trawls planned. In that these results will be pooled, statistical power would be higher for the metals and male fish as there would be greater lumped sampling size for both. Therefore, the overall objectives of the commercial fishing program and the hypothesis testing would not be compromised due to this change. A similar sampling pattern would occur at the Reference Area which is being increased in size to lessen fishing pressure in the area overall. For the Reference Area, the radius is proposed to be 8000 m to ensure enough fish are caught, especially as HMDC shares its Reference Area with the Hebron EEM Program. The Centre points for these areas are presented in Appendix A.

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Table 5-4 Summary of statistically significant results for Hibernia Platform EEM fish health indicators in American plaice compared to the Reference Area.

Indicators	2002	2004	2007	2009	2011	2014	2016	2018
Fulton's Condition Factor	No	No	No	Yes	No	No	No	No
Hepatosomatic Index (HSI)	Yes	No	No	Yes	No	Yes	No	No
Gonadosomatic Index (GSI)	Yes	No	Yes	No	No	Yes	No	Yes
Gross Pathology	No	No	No	No	No	No	Yes	Yes
Hematology	No	-	Yes	No	No	-	Yes	No
Mixed Function Oxygenase (MFO)	Yes	No	No	Yes	No	No	Yes	Yes
Liver Histopathology	No	No	Yes	Yes	No	No	Yes	No
Gill Histopathology	Yes	No	No	No	No	No	Yes	Yes

Table 5-5 Summary of statistically significant results for HSE EEM fish health indicators in American plaice.

Indicators	2011	2014	2015	2016	2018
Fulton's Condition Factor	No	No	No	No	No
Hepatosomatic Index (HSI)	No	No	Yes	No	Yes
Gonadosomatic Index (GSI)	Yes	No	No	No	Yes
Gross Pathology	No	No	No	Yes	No
Haematology	Yes	-	-	No	Yes
Mixed Function Oxygenase (MFO)	No	No	No	No	No
Liver Histopathology	No	No	No	No	Yes
Gill Histopathology	No	No	No	Yes	No

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5.3 Fish Survey Frequency

Similar to the previous versions of the plan, the commercial fish (and sediment) components of the EEM program for Hibernia and HSE will implemented every other year with the next field program currently planned for 2020.

For HWT, baseline is scheduled for the Summer of 2020 (Sediment in 2019). The Operational EEM for HWT will occur once drilling is commenced and will occur every year for the first three years. After this, and based on results, the implementation frequency may decrease.

5.4 Methods and Analysis

American plaice American plaice will be collected using an otter trawl such as a Campelen 1200 otter trawl (pictured here in Figure 5-3). Each trawl will be fifteen minutes at a trawl speed of ~3 knots. Upon retrieval of the trawl, the catch will be removed from the cod end and placed into clean plastic fish pans and sorted. American plaice will be removed first and will be placed into a flow-through holding tank maintained with fresh seawater (Figure 5-4). All other bycatch will be sorted by species, enumerated and released.

Figure 5-3 Campelen trawl being deployed for American plaice sampling

All American plaice samples will be handled using clean procedures including a complete washdown (wash with mild detergent and water, followed by isopropyl alcohol rinse and final rinse with distilled water) of the workstation and sampling instruments (scalpel, tweezers, scissors) between trawls. Personnel involved with the collection of tissue samples will use a

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new pair of nitrile gloves for each trawl and will periodically change gloves while processing fish from individual trawls.

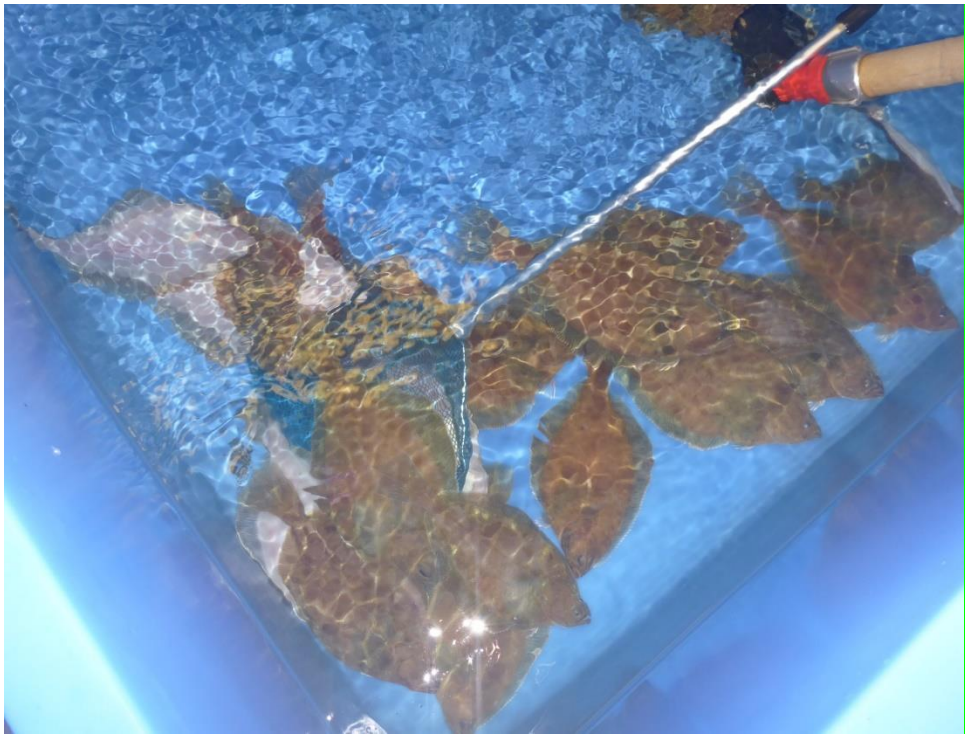

Figure 5-4: American plaice held in flow-through seawater holding tank

American plaice of appropriate size (> 25cm) will be removed from the holding tank and assigned a unique identification number. Each fish will be measured to the nearest millimeter, weighed to the nearest tenth of a gram using a motion compensating scale, and have a blood sample withdrawn for haematological analysis (bloodsmear). The fish will then be dispatched using a cranial puncture prior to the commencement of dissection. A section of the first gill arch will be removed and preserved for histopathological analysis. The fish will be dissected, the liver removed and prepared for mixed function oxygenase and histopathological analysis. Each fish will be sexed, gonads weighed, gonad maturity stage noted and the gutted weight of each fish recorded to allow the determination of a gonadosomatic index for each fish. Throughout the dissection process gross pathology and parasite loading are also assessed and noted.

Each fish will then be transferred to the filleting station where the otoliths will be removed for age analysis, the fish is filleted (skin removed) and the fillets trimmed to remove any fat. The top fillet is retained for sensory testing and the bottom fillet taken for body burden analysis.

Prior to the commencement of the sampling program, sampling kits are assembled for each individual fish. These kits are comprised of all the required sampling vessels (Whirlpak bags, Ziploc bags, coin envelopes, formalin jars) for each uniquely identified fish. The pre-labeling and assembly of fish specific kits ensures that samples are not mislabeled and that sampling errors do not occur.

The sampling numbers required are the following:

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- A minimum of 10 trawls
- 12 sets of liver composites from a minimum of 7 individuals
- 12 individual filets for body burden (metals, hydrocarbons)
- 60 fish chosen from within the group for histopathology, aging, MFO, and haematology

5.4.1 Tissue Chemical Profiles

Detailed methodologies for tissue chemistry profiles are provided in Appendix D.

5.4.1.1 Sample Preparation

Collected American plaice specimens will be immediately filleted after collection to provide for a skinless fillet (which will be portioned for various analytical requirements) and a liver sample. Samples will be stored at -20°C until analyzed.

For each sample, suitable quantities of wet tissue will be removed for the determination of dry weight and lipid content (hydrocarbons only). All analyses will be performed on wet tissue and reported on a wet weight basis. Suitable quantities will be collected from each of the samples for complete analyses for the suite of trace metals, petroleum hydrocarbons and PAHs.

Trace Metals

Homogenized tissues will be fully digested in high-purity nitric acid with sufficient digestion to ensure complete destruction of all tissues and associated fats. Determination of trace metals (see Table 5-1 for a list of metals) will be by ICP-MS with the exception of mercury which is analysed by CVAA.

Petroleum Hydrocarbons

Analyses for both petroleum hydrocarbons and a suite of 18 PAHs (see Table 5-2 for list of PAHs) will be undertaken using GC-MS, ensuring that sufficient clean up procedures have been undertaken between samples to provide for competent analyses.

For PAH determinations, a portion of wet tissue will be spiked with five deuterated surrogate PAH compounds, representing a range of volatilities, to monitor the efficiency of the sample preparation steps. The sample is extracted with 75%/25% (v/v) n-pentane/methylene chloride. An aliquot of the extract will be removed and interfering compounds are eliminated using a silica gel column clean up procedure. The extract is then solvent exchanged into iso-octane and analyzed by GC-MS using selected ion mode.

5.4.2 Sensory Evaluations (Taint Testing)

Chemical analysis of American plaice tissues that are sampled for the EEM program may not necessarily detect an overall difference in sensory perception of the sampled tissues. Therefore,

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sensory evaluations (taste tests) will be performed to determine if panelists can discriminate between study and reference area samples. Taste tests will employ the use of the triangle test and hedonic scaling as qualitative assays to determine if panelists can distinguish between study and reference area Plaice samples.

Samples will be prepared by thawing fillets for 24 hours at approximately 2°C. After thawing, fillets from each sampling area will be homogenized independently to provide uniform samples for each test. Individual samples will be prepared by placing a portion of homogenized fillet into individual foil bags, cooked in a convection oven at a uniform time and temperature and served to panelists at 35°C. Prior to cooking, each sample will be assigned a random three digit code.

Historically, the Fisheries and Marine Institute of Memorial University of Newfoundland has carried out the sensory evaluations for this EEM program. The intention is that they continue with the sensory analyses as they have the facilities, experience and have a suitable base to select panelists.

Discriminative Tests (Triangle Test)

The triangle test is a discriminatory test which involves presenting panelists with three samples of fish tissue and asking them to identify the odd sample (Figure 5-5, Figure 5-6). For this test, two samples are identical (from the same location) while the other is from another location (e.g. two from the reference area and one from a study area or vice versa). For each test panel, half of the panelists will receive two reference samples and one study area sample, while the other half of the panelists will receive two study area samples and one reference area sample. There will be four sittings of six people for a total of 24 panelists per testing session. Testing sessions will be completed to compare the Hibernia field against reference area samples.

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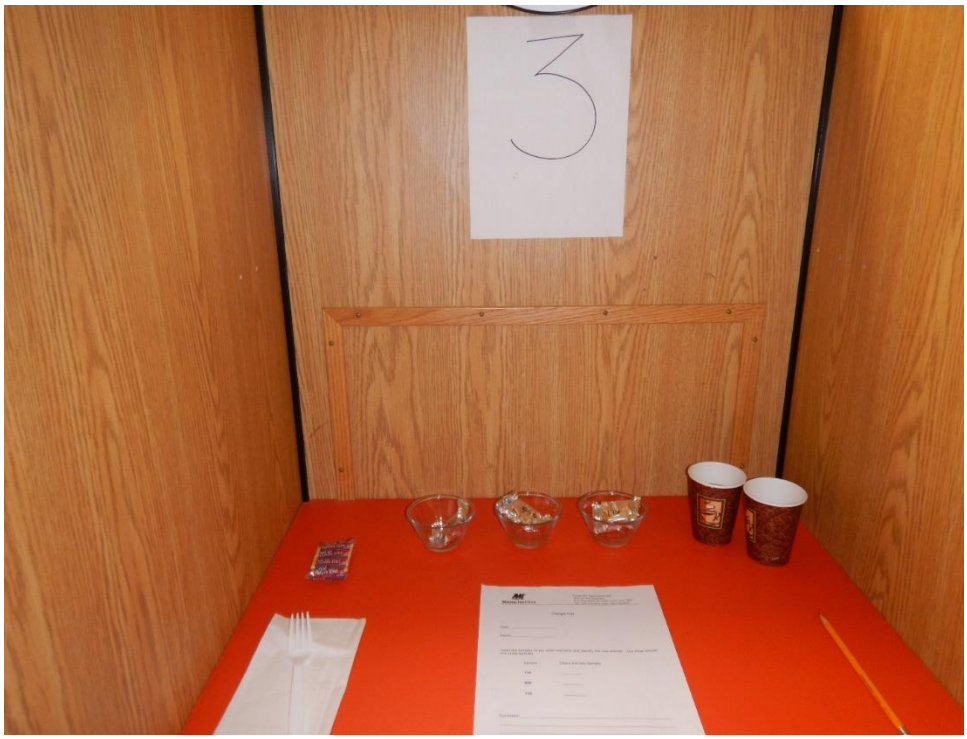

Figure 5-5: Triangle test sample presentation

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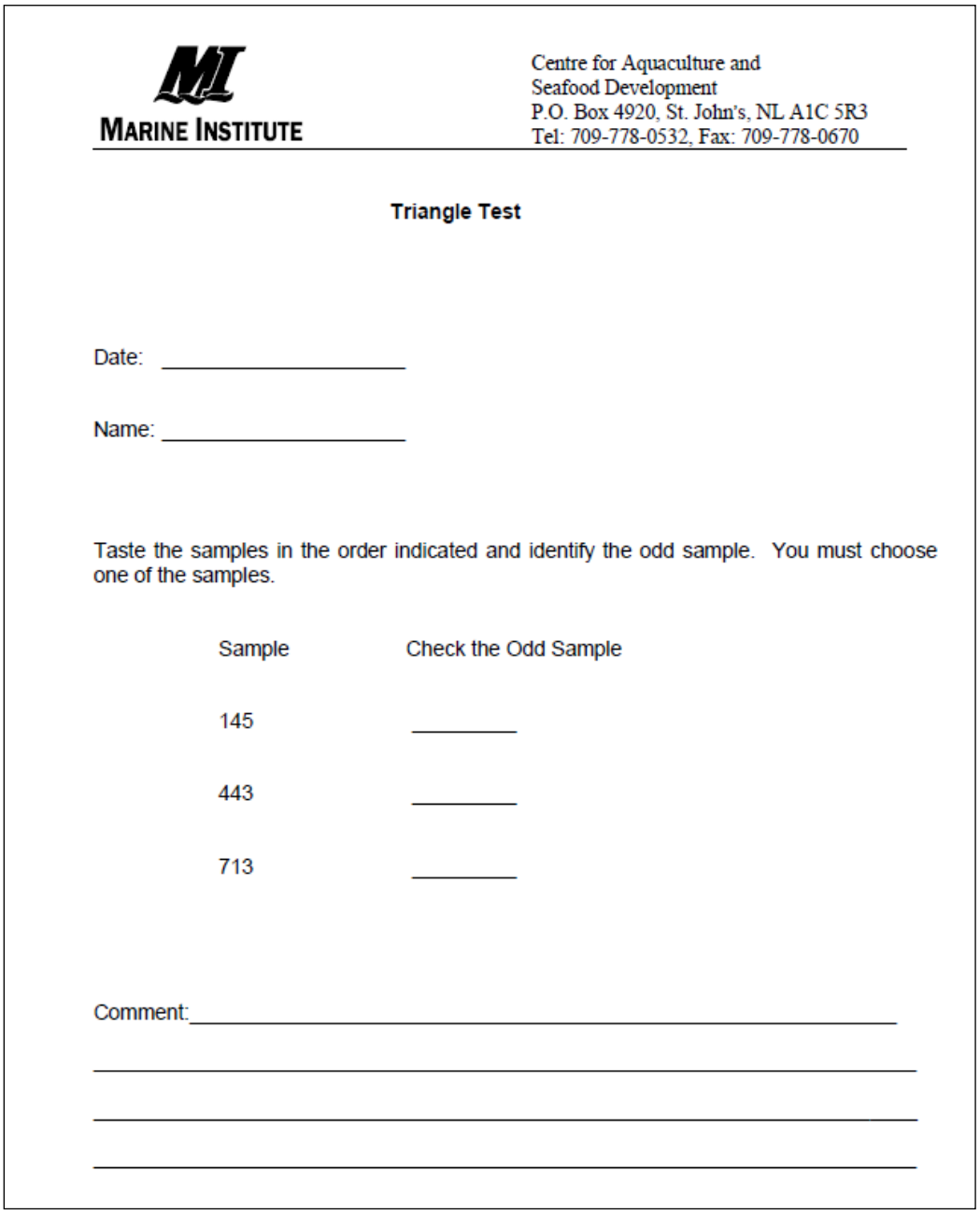

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Tel: 709-778-0532, Fax: 709-778-0670

Triangle Test

Date: _____

Name: _____

Taste the samples in the order indicated and identify the odd sample. You must choose one of the samples.

Sample	Check the Odd Sample
145	_____
443	_____
713	_____

Comment:_____

_____

_____

_____

Figure 5-6 Example Questionnaire for triangle test

Preference Tests (Hedonic Scaling)

The hedonic scaling test evaluates a panelists' preference for a sample. Panelists are presented with a sample from the study area and one from the reference area and are asked to rate each sample (Figure 5-7). Samples are rated on a nine-point scale ranging from extremely liking to extremely disliking each sample. As with the triangle test, a total of 24 panelists are used for hedonic scaling. The results are assessed using analysis of variance (ANOVA) to determine if panelists rated study and reference samples significantly different.

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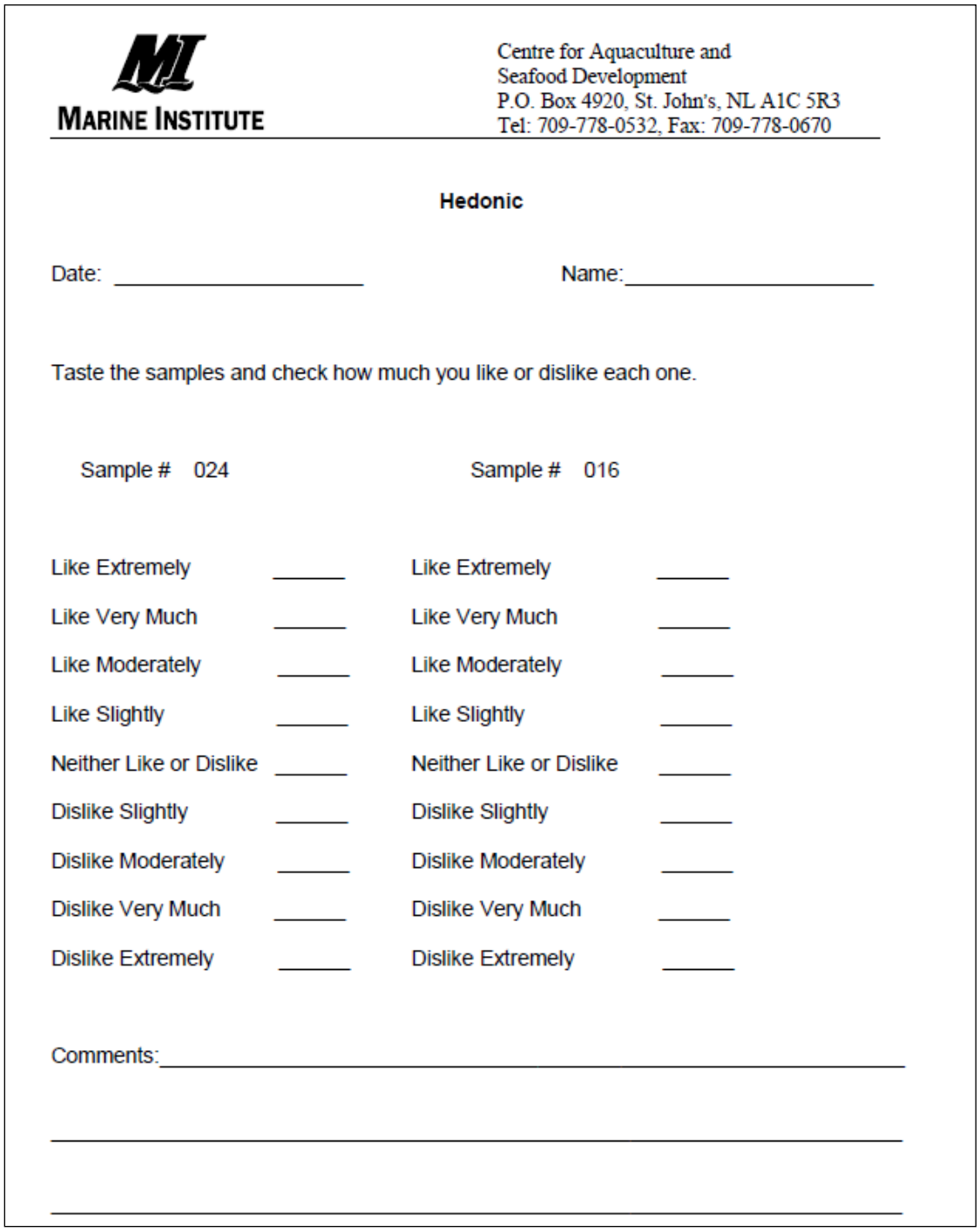

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Hedonic

Date: _____ Name:_____

Taste the samples and check how much you like or dislike each one.

Sample # 024		Sample # 016	
Like Extremely	_____	Like Extremely	_____
Like Very Much	_____	Like Very Much	_____
Like Moderately	_____	Like Moderately	_____
Like Slightly	_____	Like Slightly	_____
Neither Like or Dislike	_____	Neither Like or Dislike	_____
Dislike Slightly	_____	Dislike Slightly	_____
Dislike Moderately	_____	Dislike Moderately	_____
Dislike Very Much	_____	Dislike Very Much	_____
Dislike Extremely	_____	Dislike Extremely	_____

Comments:_____

_____

_____

Figure 5-7 Example Questionnaire for hedonic scaling

5.4.3 Fish Health Indicators

American plaice selected for analysis (Figure 5-8) will first be measured and weighed. Fish then have blood withdrawn and the animal is dispatched. All fish will be visually assessed for external parasites and abnormalities prior to dissection. Throughout each dissection, fish are also

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assessed for internal parasites and abnormalities. Each fish is sexed and gonad maturity determined according to procedures used by Fisheries and Oceans Canada (Templeman et al., 1978). The internal organs are excised with the liver and gonad weighed. The fish is also weighed to get a gutted wet weight. Fish tissues are processed as described in the following subsections.

Figure 5-8: American plaice ready to be processed

5.4.3.1 Blood

Standard haematological methods as per Platt (1969) will be used for the preparation of the blood smears. In general, blood will be withdrawn from a dorsal vein near the tail (Figure 5-9) and a pair of blood smears prepared for each fish. Blood smears will be prepared by placing a small drop of blood onto a microscope slide. A second microscope slide is then used to spread the blood into a uniform thin film. Blood smears will be air dried, fixed in methanol and then the paired blood smears will be stored in slide boxes for later analysis.

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Figure 5-9: Blood extraction from American plaice

5.4.3.2 Liver

The liver of each fish sampled will be excised and bisected. A thin slice (4-5mm thick) will be taken from the right half and stored in 10% neutral buffered formalin for histopathological processing, while the remainder of the right half will be placed into a Whirlpak bag and frozen to -80°C for mixed function oxygenase analysis. The left half of the liver is also placed into a Whirlpak bag and frozen to -20°C for body burden analysis.

5.4.3.3 Gill

The first gill arch on the dorsal side of the fish will be removed and placed in 10 percent buffered formalin for histological processing.

5.4.3.4 Otoliths

The otoliths will be removed from each fish and will be placed into a coin envelope for ageing.

5.4.3.5 Fillet (muscle)

After the otoliths are removed each fish will be filleted with the top (dorsal) fillet retained for sensory evaluations and the bottom (ventral) fillet retained for body burden analysis. Fillets are placed into Whirl pak or Ziploc bags with top fillets maintained at -26°C and bottom fillets maintained at -20°C.

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5.4.3.6 Mixed Function Oxygenase Assay

Liver samples will be maintained at -80°C in an ultralow temperature freezer while at sea. Once on shore, samples will be transferred to a cooler with dry ice and transported to the lab where they will be stored at -80°C until testing commences. Appendix D outlines the detailed sample preparation method for MFO analysis. Samples will be prepared and analyzed for ethoxyresorfin-O-deethylase (EROD) activity (considered to be the most important MFO enzyme) (Brooks et al., 2015; Hodson et al., 1991).

5.4.3.7 Haematology

Blood smears will be initially stained on board using May-Grunwald stain, followed by placement in Trizma buffer and subsequently with Giemsa stain. Slides will then be rinsed with deionized water to remove residual stain and allowed to air dry. Blood smears will then be examined using an Eclipse Ci-L microscope for the different cell types. For further reference see Fitgerald et al. (1998). Since blood cells do not disperse randomly when the smear is made the Exaggerated Battlement Method will be used to ensure that cells within a particular area of the slide are not missed. Differential blood cell counts for lymphocytes, neutrophils and thrombocytes will be completed.

5.4.3.8 Tissue Histopathology

Both liver and gill samples will be processed by standard histological methods using a Shandon Excelsior ES Tissue Processor. A graded ethyl alcohol series of 75 percent, 85 percent, 95 percent, and two changes of 100 percent will be used for dehydration of the samples. The organs will then be cleared in two changes of xylene, followed by infiltration with three changes of Histoplast PE paraffin wax. Processed tissues are embedded in steel molds with Histoplast PE wax using a HistoStar Embedding Workstation and allowed to cool and harden. The embedded tissues will be sectioned into 6µm sections using a HM 355S Automatic Microtome and floated on a 42°C water bath. Four to six sections will be placed onto a labeled microscope slide and dried on a slide warmer at 60°C for two hours. Sections will be stained using Haematoxylin and Eosin using a Varistain24-4 Automatic Slide Stainer fitted with a cover slip and allowed to air dry.

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Liver

Liver tissue samples will be microscopically examined and evaluated for lesions indicative of exposure to substances with chemical toxicity (i.e., Khan and Kiceniuk 1984; Myers et al 1990; Khan 1998, 2000). Among them are:

1. Nonspecific necrosis
2. Nuclear pleomorphism
3. Megalocytic hepatosis
4. Eosinophilic foci
5. Basophilic foci
6. Clear cell foci
7. Hepatocellular carcinoma
8. Cholangioma
9. Cholangiofibrosis
10. Increase in mitotic activity
11. Macrophage aggregates
12. Hydropic vacuolation
13. Hepatocellular vacuolation

Lesions (except macrophage aggregates) will be recorded for each fish as not detected (0) or detected (1). The percentage of fish affected by each type of lesions or prevalence of lesion will then be calculated.

Macrophage aggregation will be recorded on a relative scale from 0 to 7.

Gill

Five randomly selected gill fields of view will be examined at 200X magnification for the presence of established gill lesions as per Mallat (1985).

Five randomly selected fields will then be read for each sample at 250x magnification and examined as follows:

1. Total number of secondary lamellae will be counted and recorded.
2. Each lamella will then be examined quantitatively for six different stages of lesions:

Stage 1 - Epithelial lifting: Separation of the epithelial layer from the basement membrane.

Stage 2 – Hyperplasia

a. Distal hyperplasia: Thickening of the epithelium from the basal end and running almost the entire length of secondary lamellae .
b. Basal hyperplasia: Thickening of the epithelium near the base of secondary lamellae where they meet at the primary filament.
c. Tip hyperplasia: Thickening of the epithelium at the very tip of the secondary lamellae giving the appearance of a club.

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Stage 3 – Telangiectasis: Swelling without rupture of the capillary at the distal end of the lamellae.

Stage 4 - Fusion: Fusion of two or more lamellae.

Stage 5 - Thin lamellae – Secondary lamellae having a one-cell thick epithelial layer, with the base between two secondary lamellae having a 3-5 cell thick layer.

Stage 6 - Oedema Condition – The build-up of tissue fluid in the secondary lamellae causing swelling.

5.4.4 Quality Assurance

A sampling plan will be prepared and distributed to all field staff and relevant members of the ship's crew prior to commencing the water sampling program. Standard operating procedures outlining methods for sample collection and handling will be included in the sampling plan. All sample containers will be pre-labelled prior to departure. All pertinent information and sample specimen identification will be recorded on standard forms. Information such as length, weight, sex, maturity, biological specimen sample labels and by-catch records will be included in the forms. Storage temperatures will be monitored daily.

Upon completion of the survey, a cruise report will be issued, providing a documented history of the commercial fish survey including problems encountered. These procedures will ensure reliable and defensible data on which to base sound conclusions.

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6.0 PROGRAM IMPLEMENTATION

6.1 Health and Safety

Health and safety are an integral part of the EEM programs and it will be the contractor's responsibility to ensure that sufficient controls are in place to operate in a safe manner, thereby protecting the health of all employees. A portion of the EEM program will be conducted at sea aboard either a leased commercial or research fishing vessel (commercial fish program) or an operator-provided offshore supply vessel (sediment program). It must be recognized that while on these platforms, the ultimate responsibility and final decision for safety considerations will be at the discretion of the vessels' captains. Nevertheless, the contractor has health and safety considerations that must be considered during the execution of the EEM.

The EEM program activities are such that a hazard assessment will be undertaken prior to initiation of work. In addition to a hazard assessment, the contractor must consider safety concerns and requirements such as personal safety training and equipment, boat safety, subcontractor training and safety, fire safety, safe lifting practices and the proper use of tools. The contractor is responsible for conducting appropriate field health and safety inspections, as well as conducting daily toolbox safety meetings, to ensure safety is considered during the undertaking of all EEM activities. All safety activities should be appropriately documented.

In April of 2019 a scenario based risk assessment of the EEM program was conducted for both the Hibernia and Hebron EEM programs. The risk assessment focused on major hazards that could result in significant consequences to personnel, the environment and assets including the EEM fishing vessel. The scenarios included fishing gear entanglement with subsea equipment and dropped objects. Hazards which were identified but not associated with the scenarios reviewed were also recorded and mitigations identified. This effort resulting in updates to this EEM plan as described herein as well as the contractor SSHE plan and job hazard analysis.

6.2 Sampling Platforms

The sediment survey will be conducted from an offshore supply vessel. The vessel will typically carry two large shipment containers, one dedicated to sample processing and one (containing refrigerators) dedicated to sample storage. Other smaller shipment containers may be required to house sampling equipment. The commercial fish survey will be conducted from a leased commercial or research fishing vessel available for charter.

6.3 EEM Survey Schedule

As illustrated in Table 6-1, the core Hibernia platform, the HSE and HWT EEM program surveys will be or have been conducted every year for the first three years and then conducted every second year thereafter. Slight changes in schedule were allowed in order to synchronize the existing Hibernia and HSE EEM programs. A similar approach to synchronize HWT monitoring with the platform and HSE sampling is reflected in Table 6-1 from 2020 to 2023 in which the platform and HSE sampling is deferred by a single year. This deferral would only proceed if supported by EEM data gathered up to that time.

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The seasonal timing of the program will remain relatively consistent, when possible. The commercial fish surveys will typically be undertaken in late June-early July to maximize the probability of sufficient fish catches. The sediment survey will be typically scheduled in late July to late August to ensure limited downtime due to weather and to ensure availability of marine amphipods and polychaetes for toxicity testing. The water column program, which is conducted as part of the sediment survey, will not be scheduled for any period of planned production downtime (i.e., scheduled maintenance or turnarounds).

Baseline data will be collected for the HWT subsea development in 2019, prior to any construction. Thus, the first operational HWT EEM is currently proposed to take place in 2023 (depending on final HWT development schedule) in concert with the rest of the EEM program (Hibernia and HSE). The typical schedule is proposed to be followed with sampling at HWT every year for the first three years (up until 2025).

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Table 6-1: Past and Planned Hibernia Platform, HSE, and HWT EEM cruise schedules.

Phase	Year	Sediment Cruise Dates[1]	Biological Cruise Dates
Baseline (Hibernia)	1994	Aug. 31-Sep. 10	Dec. 04-06
Year 1 (Hibernia)	1998[2]	Aug. 26-Sep. 01	Dec. 16-23
Year 2 (Hibernia)	1999[3]	Jul. 25-30, Sep. 01-Oct. 04	Jun. 08-10
Year 3 (Hibernia)	2000	Jul. 8-18	Jul. 05-06
Year 4 (Hibernia)	2002	Jul. 06-14	Jun. 29-01
Year 5 (Hibernia)	2004	Aug. 22-28	Jul. 13-14
Year 6(Hibernia)	2007	Aug. 17-29	Jul. 17-20
Year 7 (Hibernia)	2009	Jul. 30-Aug. 04	Jun. 30-Jul. 03
Year 8 (Hibernia) Baseline (HSE)	2011	Aug. 01-06	Jul. 07-12
Year 9 (Hibernia) Year 1 (HSE)	2014[4]	Aug. 07-17	Jun. 20-Jul. 01
Year 2 (HSE)	2015	Sep. 10-16	Jun. 28-30
Year 10 (Hibernia) Year 3 (HSE)	2016	Aug. 30-Sep. 6	Jun. 25-Jul. 04
Year 11(Hibernia) Year 4 (HSE)	2018	Jul. 19-Jul. 30	Jun. 29-Jul. 06
Baseline (HWT)	2019	TBD (Aug/Sep)	TBD (Jun/Jul)
Year 12 (Hibernia) Year 5 (HSE)	2020	TBD (Aug/Sep)	TBD (Jun/Jul)
Year 13 (Hibernia) Year 6 (HSE) Year 1 (HWT)	2023[5]	TBD (Aug/Sep)	TBD (Jun/Jul)
Year 2(HWT)	2024	TBD (Aug/Sep)	TBD (Jun/Jul)
Year 14 (Hibernia) Year 7 (HSE) Year 3 (HWT)	2025	TBD (Aug/Sep)	TBD (Jun/Jul)

Notes:
[1] The sediment cruise for Hibernia sampling includes water sampling
[2] The 1999 sediment cruise was split into two cruises due to equipment losses and manufacturing of new equipment
[3] Hibernia began production in November of 1997
[4] Drilling at HSE commenced in January 2014 (HMDC, 2017)
[5] Initial drilling at HWT is planned for late 2022

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6.4 Field Documentation

6.4.1 Hazard Assessment and Safety Considerations

Hazard identification/analysis should be carried out prior to commencing the Hibernia EEM program. It is not possible to manage risks if the hazards themselves have not been identified. The identification of health and safety hazards applies to all existing activities, projects and services, as well as planned activities and services. This assessment applies to all occupational health and safety risks associated with work activities conducted by EEM contractors.

Such risks include, but are not limited to:

- activities of all personnel having access to the workplace (including subcontractors);
- all facilities at the workplace owned by the contractor or other participants in a project; and
- routine and non-routine activities.

The workplace for offshore programs would be considered to include mobilization/ demobilization locations, activities occurring at the shore-base facility and activities aboard the vessels from which the offshore EEM programs are conducted. EEM contractors are responsible to:

- identify hazards in the workplace with the assistance of their employees;
- document and address any hazards reported by their employees;
- report observed or reported hazards to the appropriate personnel;
- initiate and participate in a Hazard Assessment on existing work tasks/jobs;
- determine which hazards present significant risks of causing adverse health effects and safety concerns; and
- use Hazard Assessment practices, where necessary, to identify hazards and identify control measures and safe work practices to eliminate or control the hazards prior to starting a work task/job.

The following steps will be used to identify health and safety hazards associated with the contractor’s activities for EEM programs.

- list activities, services and products undertaken or provided by the core EEM contractor;
- identify potential health and safety hazards for each activity with consideration for work completed for the core EEM program;
- identify the controls currently in place to mitigate the hazards;
- rank each hazard in order of priority, based on the magnitude of the potential effect and the feasibility of its occurrence and its history of occurrence;
- identify significant hazards based on rank; and
- identify the legal and other requirements that directly relate to the identified hazards.

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These identified steps serve as a guide to Hazard Assessment and safety considerations that must be considered when conducting the core EEM program. The contractor may have a Hazard Assessment in place that has different steps than those outlined above. Contractors should use their system in consultation with Hibernia to conduct a Hazard Assessment for the core EEM program activities.

6.4.2 Cruise Plan

Cruise plans will be developed or updated, as appropriate, prior to the start of EEM field surveys. Cruise plans will provide the overall plan of activities for the field surveys and contain specific information regarding field crew, sample locations, location coordinates, samples to be collected, QA procedures, safety considerations and priorities for the survey. Cruise plans are intended as a general overview of the anticipated field program for use by Hibernia operations personnel, the vessel crew and the field survey team. There are two cruise plans, specific to the sediment/water program and the commercial fish program.

6.4.3 Cruise Report

Cruise reports will be developed once the EEM commercial fish and sediment field surveys are complete. Cruise reports will document the collection of samples by providing a summary of the field surveys, including vessel, personnel, mobilization, survey coordinates, a detailed report of the field survey activities and safety activities. The cruise reports will also append the sediment sample log, core description log, positioning report, daily field reports, any incident reports (e.g., damaged equipment, survey crew member injury), tow start and finish coordinates, biological data sheets and safety meeting minutes. There will be two cruise reports, specific to the sediment/water program and the commercial fish program.

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7.0 EEM REPORTING AND REVIEW

7.1 Reporting

Data collected during the EEM will be statistically compared against baseline characterization data and previous years' data for each subsequent EEM survey. The data will be reported in an interpretative document in a plain language format, to the extent possible, to facilitate the usefulness of the EEM program. The report will contain the following basic elements that are organized into chapters:

- an executive summary that will provide a general overview of the report;
- an introduction that will provide an overview of the project setting, project commitments, EEM objectives and an outline of the evaluation and interpretation of the particular years program;
- a detailed description (including tables and figures for metered discharges) of the regulated/approved discharges including produced water, storage displacement water, platform drainages, seawater return discharge, sanitary and domestic wastes and drill cutting discharges;
- a synopsis on the sediment chemistry program, including data collection, data screening, data organization and a summary of relevant chemistry data;
- the statistical analyses of the sediment chemistry data;
- details on the sediment chemistry program, including the toxicity tests results and the associated statistical analyses;
- details on the water quality program, with recommendations as appropriate;
- details on the commercial fish program, encompassing tissue chemical profiles and sensory evaluations (taint tests) that includes summary information on fish catches, tissue chemical data, statistical analyses associated with tissue chemical data and results of the sensory evaluations (taint testing);
- details on the results of the fish health program, including statistical analyses;
- a detailed discussion and interpretation of the data that discusses each of the components separately and an integrative assessment of the program that discusses the hypothesis and impact and model predictions;
- a conclusion that will highlight key results; and
- recommendations that will identify opportunities for improvement in the program.

7.2 Statistical Design

Statistical analyses and interpretations are an integral component of any EEM program and are given detailed consideration in the design of this program. The program and the data have evolved such that the method of analyses and interpretations conducted for the baseline and earlier production phase programs do not necessarily contain sufficient robustness to adequately interpret and describe the existing dataset. The statistical methods that are recommended for the

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analyses and interpretations of future Hibernia EEM programs are detailed below. Statistical methods are a component of the EEM program that requires continued assessment for relevancy and will be subject to review after each year's data are collected and analysed. The current methods are listed here for context but may be revised based on a review of data and as better methodologies evolve.

7.2.1 Hypothesis

The design of the EEM program monitors critical elements of the receiving marine environment to provide timely and beneficial information to detect deleterious effects to the marine environment. The program has been strengthened based on recommendations from regulatory review as well as experience gained during previous Hibernia EEM programs. The body of data that is collected from biological and sediment monitoring programs will be evaluated in a scientifically-defensible manner (based on statistical analysis and interpretation with peer-reviewed research) for early indications of potential project-induced alterations to the surrounding environment for which a series of hypotheses have been developed.

The monitoring hypotheses for the EEM program are consistent between the three programs (Hibernia Platform, HSE, and HWT) are as follows:

Hypothesis No.1:

- H_0 = Approved releases of solids and liquids from Operations will not result in significant adverse environmental effects on marine fish (as assessed by fish health indicators and integrative assessment).
- H_A = Approved releases of solids and liquids from Operations will result in significant adverse environmental effects on marine fish (as assessed by fish health indicators and integrative assessment).

Hypothesis No.2:

- H_0 = Approved releases of solids and liquids from Operations will not result in significant adverse environmental effects on marine fish habitat (as evaluated by sediment toxicity assays and integrative assessment).
- H_A = Approved releases of solids and liquids from Operations will result in significant adverse environmental effects on marine fish habitat (as evaluated by sediment toxicity assays and integrative assessment).

Hypothesis No.3:

- H_0 = Approved releases of solids and liquids from Operations will not result in the taint (as measured by organoleptic evaluations and integrative assessment) of fishery resources outside the safety zone.

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- H_A = Approved releases of solids and liquids from Operations will result in the taint (as measured by organoleptic evaluations and integrative assessment) of fishery resources outside the safety zone.

  For Hibernia Platform 'Operations' is defined as the Hibernia Platform's production and drilling operations. For HSE and HWT, 'Operations' is defined as drilling operations. Due to changes in the fish trawling (see Section 5.2), for Hypothesis No. 1 and 3, the operations will be assessed as a whole-field analysis rather than individual operations.

To assess the environmental effects of the projects, an integrative weight of evidence approach is used whereby the physical, chemical, toxicity and biological data are considered collectively; thereby providing a more informed interpretation of the potential ecological impacts derived from chemical (pollutant) or other stressors (Chapman, 2007). Essentially, the results of the analyses from each component of the EEM program are reviewed collectively to provide an overall interpretation of the program hypotheses. For example, the null hypothesis for No. 2 would be rejected if the outcome of the EEM program were to demonstrate:

- Biological toxicity testing (including Petrotox sediment quality guideline and/or amphipod/polychaete toxicity) indicate a toxic response at a sediment sampling station in the vicinity of the project;
- The toxic response is correlated to the elevated presence of other analytes within the sediment known to be associated with drilling operations;
- There is a statistically significant difference in the concentration of that analyte within the sediment compared to background or baseline conditions.

Where a biological effect beyond the predictions of the Environmental Impact Statement is confirmed, the determination of biological significance of this effect according to the hypothesis statements of the program (listed above) will be included in the annual environmental assessment update. These interpretations will include a qualitative and where possible, quantitative assessment using existing knowledge (referenced scientific studies), professional judgement and analytical tools.

Should a biological effect beyond the predictions of the environmental assessment be identified through the EEM program, the sampling associated with the program will be reviewed and, if needed, adapted to ensure resolution is adequate to determine the spatial scale of any observed exceedance. Any adaptations would be subject to the approval of the C-NLOPB. The subsequent EEM program would proceed as per its regular schedule (unless a change is agreed to by the C-NLOPB) and include modifications to the sampling program design.

7.2.2 Sediment Chemistry Statistical Analyses

A detailed review of the sediment chemistry analysis was undertaken in 2003 and resulted in focusing statistical analysis primarily on barium and hydrocarbons. Other chemical parameters that remained unchanged since the baseline program in 1994, while collected as part of the suite of trace metals, will no longer be subject to statistical analyses.

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Similarly, the continued use of statistical analyses for parameters that have returned or encroached upon baseline levels in 2002 and that remained at similar or lower levels since 2004 is not necessary for future EEM programs, provided current operating conditions remain constant. The data collected in 2004 indicated most contaminant levels have returned to 1998 or baseline levels and therefore, the application of statistical analyses is not relevant. Statistical analyses will be applied when there exists an obvious deviation from the baseline, or operations sampling data.

The first step in data analyses and interpretation is the screening of data to select chemical parameters for further statistical analysis in the event of a reversal to the improving trend observed in the previous EEM programs (as assessed by the two-dimensional (2-D) plots). In order to assess the spatial distribution of the chemistry results, 2-D plots will be developed using R or a comparable statistical package. The spatial and temporal trends of sediment chemistry are presented in 2-D surface bubble plots with the X and Y axes representing the east-west and north south distances respectively (in meters). For Hibernia, the X and Y axes scales have been set to 6000 m with 1000 m scale plots for certain analytes where greater resolution is needed for near-field distribution patterns. For HSE and HWT, the X and Y axes scales will be set to 1000 m for greater resolution of the Near-field. An example of the 2-D surface plots (without sample grid) that will be used to illustrate the relevant chemical parameters is provided in Figure 7-1.

Following the visual screening, examination of the summary data and above-noted criteria, a determination will be made on which parameters to carry forward into exploratory data analysis and statistical analysis. In cases where data values below the estimated quantitation level (EQL) are present for a substance that was the subject of plotting or further statistical evaluation, the “non-detectable” observation will be treated using ½ EQL as per previous agreements. Updated EQLs will be used where appropriate.

The statistical approach to evaluating monitoring data has been structured to consider two levels of scale. The first is a coarse screening for differences between Near-field (stations between 250 and 1,000 m from the Platform), Mid-field (stations between 1,500 and 3,000 m from the Platform), and Far-field (4,000 to 6,000 m from the Platform) stations. The division of stations into Near-, Mid- and Far-field groupings is arbitrary, but reflects the varying spatial extent of effects predictions developed in the Hibernia production phase EEM program (HMDC 1996). The second statistical analysis focuses on effects within 1,000 m of the Platform, by looking explicitly at effects at the distance radii of 250, 500, 750 and 1,000 m. In both cases (coarse- and fine-scale analysis), the analysis explicitly tests for effects attributable to distance from the Platform (either as Near-, Mid- or Far-field, or explicitly as distance), the year of the EEM program (time) and, most importantly, the potential interaction term between distance and time.

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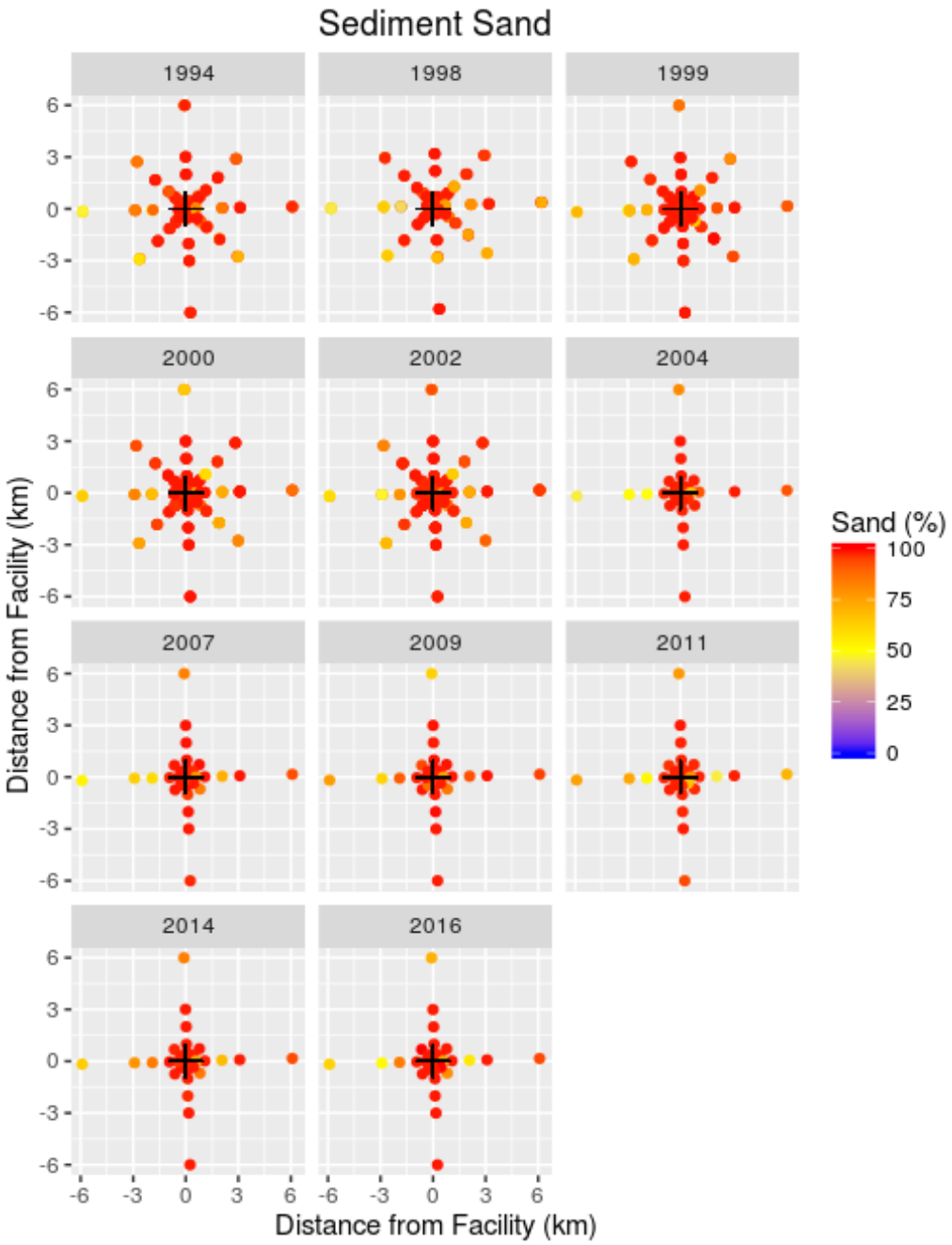

Figure 7-1 Example of a Two-dimensional Surface Plot: Average Sand Content in Hibernia Sediment (1992-2016)

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The use of repeated sampling over time also results in a technical violation of the assumptions of ANOVA, since observations at any single station sampled over time can be said to be auto-correlated (in effect, they tend to be more similar to observations from the previous and subsequent sampling periods than they should be if they were statistically independent samples). In spite of these difficulties, as long as it is recognized that a relatively high incidence of “false positive” observations can be expected, the technique remains an important data interpretation tool. Other statistical tools, such as linear mixed models may be used to account for some of these concerns. In addition, in order to aid in interpreting the importance of statistically significant differences, and to help screen out “false positive” results, four principles can be considered. These are the principles of:

- proximity (differences are more likely to be real if they are observed in close proximity to the source of the disturbance);
- adjacency (differences are more likely to be real if two or more adjacent locations are affected);
- multiplicity (differences are more likely to be real if two or more parameters are affected simultaneously); and
- duration (differences are more likely to be real if they persist over time).

Although each of these principles is evaluated independently, they can be taken cumulatively to establish a weight-of-evidence, suggesting that observed statistically significant differences either are, or are not, true effects of the project.

The following chemical parameters will be subjected to statistical analysis using ANOVA: total barium; weak-acid extractable barium; fuel range hydrocarbon; and lube range hydrocarbon. Each of the chemical parameters will be analyzed in two steps. The first step is coarse-scale analysis, where the various distances are divided into Near-, Mid- and Far-field groups. All years for which data are available will be processed. At each sampling station, the mean value for the available data for each year will be taken as the best point estimate. In instances were only one data point is collected per station (post-2002 Hibernia EEM programs), that data point will be used. Note that in this statistical analysis, the sampling station is the experimental unit. The use of individual samples collected at each station is therefore not valid, since these samples are not true replicates, but should be viewed as sub-samples. A statistical error term calculated using the sub-samples as if they were true replicates would underestimate the inherent variability between stations and would therefore further inflate the rate of false positive results. The second step in the analysis is to examine the near-field stations, and to look for effects attributable to fine-scale Distance (250, 500, 750 or 1,000 m) or Year, where the effects are most likely to be observed.

The HSE and HWT EEM will rely on the same methodology for sediment chemistry statistical analyses as per the core EEM program as described herein. Changes in sediment chemistry will be tested using a one way ANOVA design along a distance gradient (factor levels: 250, 500, 1000 and 16,000 m). The analysis will include an analysis of the core, HSE, and HWT data sets separately and as a combined data set.

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7.2.3 Sediment Toxicity Statistical Analyses

Toxicity tests will be analyzed and interpreted in a two-step process. The first step will be the interpretation of the toxicity test based on guidelines developed by Environment Canada for the determination of toxic/non-toxic responses. The second step is a more detailed statistical analysis similar to that used for the sediment chemical statistical analyses.

To assess if the toxicity results correlate with sediment chemistry data for Hibernia, HSE, or HWT, Pearson correlation coefficients will be calculated using log(10) transformations on concentration data and square-root arcsine transformations on percentage data (e.g., percent sediment particle size and amphipod survival). Transformations will be applied to promote the normality of the data and homogeneity of the variance for parametric assumptions of the statistical analysis. A correlation coefficient of +1.0 would indicate that there was a perfect linear relationship between two variables, with both values increasing or decreasing together. A high correlation coefficient does not signify a causative or dependent relationship between the two parameters being examined, where one parameter is responsible for the outcome of the other parameter. A correlation coefficient of -1.0 would indicate a perfect inverse relationship between the two parameters, such that the value of one parameter increases as the other decreases. Correlation coefficients rarely equal 1, but always lie between +1.0 and -1.0. In this report, a Pearson correlation coefficient ("r" value) having an absolute value of 0.55 or greater is arbitrarily considered to be meaningful, and worthy of discussion.

A single-factor ANOVA using the general linear model will be used to determine whether significant differences in sediment toxicity data could be detected between Near-, Mid- and Far-fields from the Hibernia, HSE, or HWT. This first coarse-scale analysis is based on an arbitrary division of the various station distances along the radials into Near-, Mid- and Far-field groups. Where a significant difference attributable to the experimental factor field is indicated in the ANOVA, the source of this difference will be investigated using Tukey's HSD multiple comparison tests. Percent amphipod survival data will be square-root arcsine transformed prior to analysis to ensure the assumptions of the ANOVA analyses are met. In 2002, there were insufficient data for amphipod survival, to obtain the minimum degrees of freedom necessary to test the interaction effect of Year X Field in the ANOVA. Therefore, for the 2002 data, only Year and Field were tested for statistical significance in the ANOVA. It is plausible this may occur for future data and, as for the 2002 data, only Year and Field will be tested for statistical significance in the ANOVA in the event of such an occurrence.

7.2.3.1 Amphipod Survival Interpretation Guidelines

The Amphipod Survival Assay endpoint is percent mortality. The statistical endpoint for the amphipod toxicity test is the determination of whether the biological endpoint (percent survival) differs statistically from the control or reference sample, calculated using a t-test such as Student's or Dunnett's. The amphipod survival test results will be considered toxic if the endpoint (mortality) is more than 30 percent higher and significantly different than mortality in the negative control sediment (sediment collected from the amphipod collection site) at the p=0.05 level. The Amphipod Survival Test results would be considered toxic if the endpoint (mortality) is more than

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20 percent higher and significantly different than mortality in the Reference sites (16 km Hibernia Reference sites) at the p=0.05 level.

7.2.3.2 Juvenile Polychaete Growth

The statistical endpoint for the juvenile polychaete toxicity test is the determination of whether the biological endpoint (percent survival and mean growth) differs statistically from the control or reference sample, calculated using TOXCALC computer program (Tidepool Scientific Software 1994). Environment Canada has no interpretative guideline for the juvenile polychaete toxicity test. For the purpose of this report and as an aid for the interpretation of the results, the amphipod survival tests interpretative guidelines will be used as a guide for the interpretation of the juvenile polychaete data. Therefore, the juvenile polychaete survival test results would be considered toxic if the endpoint (mortality) is more than 30 percent lower and significantly different than mortality in the negative control sediment at the p=0.05 level. The juvenile polychaete growth test results would be considered toxic if the endpoint (mean growth) is more than 30 percent lower and significantly different than mean growth in the negative control sediment at the p=0.05 level.

Alternatively, the juvenile polychaete survival test results would be considered toxic if the endpoint (mortality) is more than 20 percent lower and significantly different than mortality in Reference site sediment at the p=0.05 level. The juvenile polychaete growth test results would be considered toxic if the endpoint (mean growth) is more than 20 percent lower and significantly different than mean growth in the Reference site sediment at the p=0.05 level.

7.2.4 Water Quality Analyses

The water quality program will be undertaken to determine whether information can be collected within the water column on hydrocarbons via standard laboratory analyses. The purpose of the water quality monitoring is to validate the produced water dispersion modelling and to assess the extent of a potential zone of effects. The water quality data analyses will include a comparison of predicted and actual dilution factors.

7.2.5 Statistical Analyses of Tissue Chemistry Profiles

Statistical tests (ANOVA) will be performed on the biological tissue data to evaluate whether there are significant differences in the chemical concentrations present in liver or muscle tissues of American plaice, between fish collected in the vicinity of the Hibernia field (Hibernia Platform, HSE, or HWT) and those collected at a Reference site. For the core EEM program, only the data collected since 1994 will be used for the analysis, since the data collected in 1994 did not include sufficient fish to support statistical analysis.

The first step of analysis is to screen the data and select for further analysis only those parameters that are reliably detected. If data are routinely below the EQL (>50%), then no further analysis will be conducted. Based upon the screening, selected parameters will be retained for statistical analysis of muscle and liver tissues. In cases where data values below the EQL are occasionally present for a parameter that is the subject of further statistical evaluation, the “non-detectable” observation will be replaced in the data set by a numerical value equal to one-half the EQL.

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A two-way ANOVA using the general linear model will be used to determine whether statistically significant differences in chemical concentration are present in American plaice liver and muscle tissues caught near the Hibernia field (Hibernia Platform, HSE, or HWT) and from a Reference site. The experimental factors or treatments that will be statistically tested in the ANOVA include Year and Area (Hibernia field and Reference), as well as the Year X Area interaction term. Where statistically significant differences attributable to either of the experimental factors or interaction term are indicated in the ANOVA, the source of these differences will be investigated using Tukey's HSD multiple comparison tests. For all statistical testes (unless noted otherwise), comparisons between sites having a $p<0.05$ are considered to be statistically significant.

7.2.6 Statistical Analyses for Sensory Evaluations (Taint Testing)

The statistical methods employed for organoleptic (taint testing) evaluations range from standard tables to vigorous parametric analyses. The statistic method used will depend on the actual organoleptic assessment technique.

A determination of taint necessitates that a statistical difference for the triangle test and an unacceptable ranking for the hedonic scaling have been obtained.

7.2.6.1 Triangle Test

The triangle test datum is a value that represents the number of correct responses over the number of panelists. This value is compared to values in a standards table to determine the statistical significance of the result. A statistically significant result for the recommended panel size of 24 would require 13 correct responses (95 percent significance level).

7.2.6.2 Hedonic Scaling

Hedonic scaling assesses the magnitude of preference along a nine-point scale. The scale lends itself to the assignment of values (1 to 9) and statistical analysis of the data by ANOVA. Samples from the Hibernia and Reference sites are analyzed to determine if there is a statistical difference within a sample or between the two samples. The drawback to the use of the ANOVA is the assumption that each point of the scale is of equal size and value. Nevertheless, the ANOVA analysis allows for the detection of differences between sample locations while accounting for differences attributable to sample variability. Graphical presentation in the form of a frequency histogram aids in the interpretation and understanding of hedonic scaling results and should be used as an illustrative aid.

7.2.7 Statistical Analyses of Fish Health Indicators

7.2.7.1 Mixed Function Oxygenase Induction

Enzyme activities in liver tissues of fish of the same sex from the Hibernia field Reference sites will be compared by one-way ANOVA or Mann-Whitney Rank Sum test when data are not normally distributed. Cross-year comparisons will be made using two-way ANOVAs comparing the asset areas (Hibernia/HSE/HWT) and Reference Area across years for each sex.

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7.2.7.2 Haematology

A blood cell differential count will be performed on lymphocytes, neutrophils and thrombocytes and expressed as a percentage of each type of cells for 200 cells counted on each slide. Percentages will be analyzed by one-way ANOVA or the Mann-Whitney Rank Sum test when data are not normally distributed (Sokal and Rohlf 1981). Cross-year comparisons will be made using two-way ANOVAs comparing the asset areas and reference area across years for each sex.

7.2.7.3 Tissue Histopathology

In livers, the macrophage aggregation will be recorded on a relative scale from 0 to 7 and prevalence will be calculated for fish showing a moderate to high aggregation (3 or higher on the scale). Prevalence of lesions will be statistically analyzed by the Fisher's exact test.

For gill tissue, the results for each fish will be expressed as the percentage of lamellae presenting the stage in relation to the total number of lamellae counted in the fields. Percentages will be analyzed by one-way ANOVA (Sokal and Rohlf 1981).

For both liver and gill results, cross year comparisons will be made using multivariate analysis of variance (MANOVA). This test will incorporate all factors affecting fish for each tissue and compare theHibernia field and reference area across years. Each factor in the MANOVA with be analyzed by two-way ANOVA to determine which factors, if any, are driving significant results in the MANOVA.

The degree of oedema present in gills, if any, will be recorded on a 0 to 3 relative scale (0-absent, 1-light, 2-moderate and 3-heavy) and compared among sites with a one-way ANOVA or Mann-Whitney Rank Sum test when data are not normally distributed.

7.3 Decision Making

The EEM program is one component in Hibernia's environmental management system (OIMS System 6-5 Environmental Management). The EEM program provides HMDC with the necessary information to make project-related decisions with respect to environmental practices associated with effects on the marine environment.

7.4 EEM Program Review

The EEM program will be reviewed after each year that data are collected. Each of the steps in the program including the sample grid designs will be evaluated and, if necessary, refined to better meet the objectives of the EEM. Any recommended revisions will be submitted to C-NLOPB for approval prior to any alteration to the program.

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APPENDIX A

Coordinates for Sampling Stations

(NAD 83, Zone 22, UTM)

(NAD 27 included for comparison; converted using NTv 2)

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Table A-1. Hibernia Sediment Stations Coordinates

Station ID	NAD83 UTM Zone 22		NAD27 UTM Zone 22		Location	Notes
	Easting	Northing	Easting	Northing		
2-250	669589	5180133	669512	5179922	HIB	2019 Change due to 100-m buffer
4-250	669667	5179802	669589	5179591	HIB	2019 Change due to 100-m buffer
5-500	669341	5179317	669264	5179106	HIB	2019 Change due to 100-m buffer
5-2000	669403	5177811	669325	5177600	HIB	2019 Change due to 100-m buffer
6-250	669439	5179562	669361	5179351	HIB	2019 Change due to 100-m buffer
6-500	669038	5179489	668961	5179278	HIB	2019 Change due to 100-m buffer
7-500	668959	5180012	668882	5179802	HIB	2019 Change due to 100-m buffer
7-1000	668417	5179829	668340	5179619	HIB	2019 Change due to 100-m buffer
7-6000	663422	5179585	663345	5179374	HIB	2019 Change due to 100-m buffer
8-250	669278	5180148	669201	5179937	HIB	2019 Change due to 100-m buffer
8-500	669105	5180202	669028	5179991	HIB	2019 Change due to 100-m buffer
1-500	669404	5180315	669327	5180104	HIB	
1-1000	669389	5180809	669312	5180598	HIB	
1-2000	669361	5181814	669284	5181603	HIB	
1-3000	669333	5182814	669256	5182603	HIB	
1-6000	669249	5185813	669172	5185602	HIB	
1-16000 (a,b)	668966	5195808	668889	5195597	HIB	
2-500	669757	5180147	669680	5179936	HIB	
2-1000	670105	5180542	670028	5180331	HIB	
3-500	669918	5179829	669841	5179618	HIB	
3-1000	670414	5179843	670337	5179632	HIB	
3-2000	671417	5179872	671340	5179661	HIB	
3-3000	672417	5179900	672340	5179689	HIB	
3-6000	675415	5179984	675338	5179773	HIB	
4-500	669782	5179471	669705	5179260	HIB	
4-1000	670145	5179128	670068	5178917	HIB	
5-1000	669446	5178810	669369	5178599	HIB	
5-3000	669502	5176817	669425	5176606	HIB	
5-6000	669587	5173818	669510	5173607	HIB	
6-1000	668731	5179088	668654	5178877	HIB	
7-2000	667419	5179759	667342	5179548	HIB	
7-3000	666419	5179731	666342	5179520	HIB	

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Table A-2: HSE, HWT, and Fishing Reference Station

Station ID	NAD83 UTM Zone 22		NAD27 UTM Zone 22		Location	Notes
	Easting	Northing	Easting	Northing		
N-1000	672081	5175098	672004	5174887	HSE	
N-500	672091	5174595	672014	5174384	HSE	
N-250	672096	5174338	672019	5174127	HSE	2019 Change due to 100-m buffer
E-1000	673019	5174052	672942	5173841	HSE	2016 Change for current meter
E-500	672516	5174035	672439	5173824	HSE	
E-250	672266	5174052	672188	5173841	HSE	
S-1000	671967	5173101	671890	5172890	HSE	
S-500	671952	5173605	671875	5173394	HSE	
S-250	671945	5173861	671868	5173650	HSE	
W-1000	671022	5174157	670944	5173946	HSE	2019 Change due to 100-m buffer
W-500	671520	5174114	671443	5173904	HSE	2019 Change due to 100-m buffer
W-250	671818	5174248	671741	5174037	HSE	2019 Change due to 100-m buffer
6000-1	675397	5179055	675320	5178844	HSE	
6000-2	678018	5174048	677941	5173837	HSE	
6000-3	677461	5171574	677384	5171363	HSE	
6000-4	676231	5169829	676154	5169618	HSE	
2NE-250	664737	5179323	664660	5179112	HWT	At 275m due to 100-m buffer
2NE-500	664856	5179401	664779	5179191	HWT	
2NE-1000	665270	5179682	665193	5179471	HWT	
1N-250	664497	5179350	664420	5179139	HWT	
1N-500	664533	5179598	664455	5179387	HWT	
1N-1000	664627	5180089	664550	5179878	HWT	
4W-250	664208	5179147	664130	5178936	HWT	
4W-500	663962	5179191	663884	5178980	HWT	
4W-1000	663469	5179278	663392	5179067	HWT	
3S-250	664411	5178858	664334	5178647	HWT	
3S-500	664367	5178612	664290	5178401	HWT	

Table A-3: Fishing Reference Area

Station ID	NAD83 UTM Zone 22		NAD27 UTM Zone 22		Location	Notes
	Easting	Northing	Easting	Northing		
Fishing Reference	634985	5215833	634908	5215622	REF	8-km radius

APPENDIX B

Sediment Chemistry and Toxicity Methods

METHOD SUMMARY

Analysis of Ammonia (plus Ammonium) – Nitrogen in Aqueous Samples

SOP #: ATL SOP-00015

Reference: USEPA Method 350.1 Determination of Ammonia Colourimetric, Automated Phenate)

1. Scope and Application

Ammonia – Nitrogen includes both Ammonia (NH_3) and Ammonium (NH_4^+). This method is applicable to the analysis of surface waters, ground waters and saline waters. Industrial and sewage wastewaters can be analyzed as long as they are not highly coloured. Soil samples may be analyzed for aqueous soluble ammonia following an aqueous extraction as per ATL SOP-00033. The Reporting Detection Limit (RDL) for water is 0.05 mg/L as N and 0.25 mg/kg as N for soils.

2. Summary of Method

An Automated Colorimetric Analyzer is used for sample and reagent handling and colour development. Samples are lab filtered and mixed with EDTA to remove interferences and alkaline phenol and sodium hypochlorite are added to react with ammonia to form indophenol blue. The absorbance of this compound at 660 nm is directly proportional to the soluble ammonia concentration.

3. Quality Assurance

Certified reference materials, method blanks, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of 5%.

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BTEX/C6-C10 AND VOC FIELD PRESERVATION, Atlantic Canada

TECHNICAL BULLETIN

Under a proposed amendment to Atlantic RBCA Hydrocarbons, a significant change will occur for collection of soil samples for VOCs and BTEX/C6-C10 petroleum hydrocarbons in early 2016. (http://www.atlanticrbca.com/). This technical document provides a description of sampling methods available.

Field Preservation:

Field preservation is the preferred sampling procedure for the following reasons:

- ⇨ Hold times for VOCs and BTEX/C6-C10 PHC field preserved soil samples will now be 28 days.
  Note: Extractable Petroleum Hydrocarbons (C10-C32) hold time remains at 14 days.
- ⇨ It provides the best possible data quality according to the California EPA.

It is important that special attention and care be taken when collecting and preserving samples in the field. Methanol must not leak or spill out of the vial during transportation or sampling as this will affect the weight and bias data. Threads of the vial and cap must be cleaned and free from grit prior to closing to prevent methanol from leaking out.

Maxxam Field Preservation Kits for BTEX/C6-C10 and VOCs

- **Two pre-weighed vials with 10 ml of purge and trap (P&T) grade methanol for each VOC or BTEX/C6-C10 analysis**. Two vials will allow for proper lab QC and duplicate analysis.
- One heavy duty 10 ml Terra Core™ Sampler pre-calibrated to deliver approximately 10g of wet weight soil
- One 60 ml jar for moisture (no headspace) which can also be used for C10-C32 analysis (for additional testing a 2nd jar may be required)
- Instruction sheet with MaxxTips/MSDS

Cost of kit for two vials (C6-C10 or VOCs): $4.25

Cost of kit for four vials (C6-C10 and VOCs): $6.75

BTEX/C6-C10 AND VOC FIELD PRESERVATION, Atlantic Canada

Storage / Shelf Life for Field Preservation Kits:

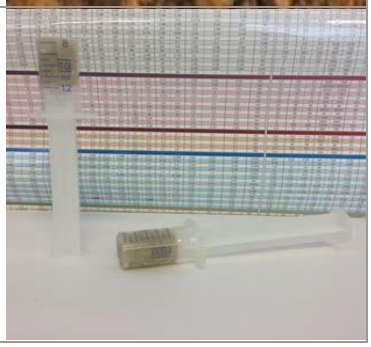
Shelf life for P&T grade methanol stored in a clean, cool, dry place with minimal temperature variations can be up to one year. It is recommended that vials be discarded if the methanol level is below the black reference line. Maxxam suggests kits be used as soon as possible. It is also recommended that clients submit a trip blank (clean vial of methanol) that has been carried through sampling and handling conditions as a contamination check.

Invoicing for Field Preservation Kits:

Field preservation kits will be invoiced as orders are placed. Customer project numbers must be provided at the time of order.

Optional coring devices for field preservation:

Though Maxxam's default will be to provide our customers with 10mL Terra Core™ samplers, we can also supply consultants with pre-cut syringes. Note that the cut-off syringes are calibrated to deliver only 5-6g so 2 cores are required for each vial. With both the Terra Core™ Samplers and cut-off syringes, one sampling device can be used for multiple vials however a new device must be used between each sampling point.

Coring Devices For Field Preservation	
Terra Core™ Sampler: - Suitable for most soil types - Sturdy construction with easy to grip handle - 1 x 10g core required per vial - One Terra Core™ used per sample; then discarded **Cost: $1.75 / unit**	
Pre-cut Syringe: - Good for loamy type soil - 2 x 5-6g cores required per vial - One syringe used per sample; then discarded **Cost: $1.00 / unit**	

For additional guidance and information, please contact your Maxxam Project Manager or Maxxam Account Manager.

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BTEX/C6-C10 AND VOC FIELD PRESERVATION, Atlantic Canada

Hermetic Samplers:

There may be situations when hermetic sampling devices are better alternatives to field preservation. This option may be preferable in some instances such as extremely hard soils or if no methanol kits are available. Keep in mind:

- **48 Hour Hold Time** (Samples need to be preserved in the lab within 48 hours from sampling).
- 4 x 5g cores are required for each analysis for laboratory QC or field duplicate.
- One 60ml jar with soil is still required to obtain moisture content; can also be used for C10-C32.
- Maxxam will require cores to be received at least four hours prior to hold time expiry to ensure sample inspection, entry and preservation can all occur within 48 hours from collection.
- Cores requiring immediate preservation may be subject to a rush preservation surcharge
- To purchase Hermetic sampling devices, place orders well in advance so Maxxam can ensure timely delivery of supplies.

Hermetic Sampling Kits Supplied by Maxxam:

- **ESS Core N' One 5 gram Capsule and Handle**
  - Handle is strong, economical and easy to use
- **Core N' One Capsule:**
  - Screw-thread Septa closure ensures air-tight seal and zero-headspace
  - Capsule incorporates extruding feature; no need for expensive tools
  - Includes 8-digit QC Lot Number from a QC Class Certificate of Compliance that confirms the Independent Analysis using Method 524.2 plus fuel oxygenates
- **One 60 ml Jar to correct for moisture**

ESS Core N' One Capsule & Handle		EnCore Sampler	ESS Core N' One
	Price for handle	$125.00	$12.50
	Handle durability	Yes	Yes
	Price / Individual Item	$8.00	$6.00
	QC Lot Batches	Not Available	Available
	Capsule attachment to handle	Cumbersome	Easy; no spring loaded latch
	Exposure of VOC artifacts	Rubber O-ring on the flange exposes VOC artifacts to soil (data available)	No rubber exposed to sample, QC checks conducted using EPA Head Space TO 15
	Core cap seal	Prongs have to be lined up properly, or seal is compromised	Screw-thread with Teflon liner provides better seal; cap remains tight

Presentations on regulatory amendments are available from Maxxam, including demonstrations on the EPA 5035A sample collection, preservation and storage protocols. Please contact your Maxxam sales or service representative or email **enviro@maxxam.ca** for further information.

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METHOD SUMMARY

Volatile Petroleum Hydrocarbons in Soil/Sediment

SOP #: ATL SOP-00119

Reference: Atlantic PIRI Guidelines for Laboratories

1. Scope and Application

This method is designed for the extraction and analysis of volatile petroleum hydrocarbons, including MTBE, benzene, toluene, ethylbenzene, o-xylene, m-xylene, p-xylene (BTEX), and gasoline range organics (C6-C10) in soils and sediments. The reporting limit is 0.025 mg/kg for MTBE and toluene, 0.05 mg/kg for total xylenes, and 2.5 mg/kg for gasoline. Low level benzene and ethylbenzene reporting limits are 0.005 mg/kg, and 0.01 mg/kg respectively.

This method is used in conjunction with SOP # ATL00111, "Total Extractable Hydrocarbons (>C10 – C32) in Soil" to quantify Total Petroleum Hydrocarbons in a sample.

2. Summary of Method

A 10 gram portion of wet soil or sediment is extracted by shaking with methanol. An aliquot of the methanol extract is diluted into water and analyzed by headspace-gas chromatography with mass spectrometry/flame-ionization detection (GC-MS/FID). A surrogate standard (isobutylbenzene) is added to the sample to monitor instrument performance.

The instrumentation is calibrated with multi-component standards of known concentration. Calibration accuracy is verified with independent reference standards of BTEX and gasoline. The day-to-day stability of the calibration is confirmed by analyzing calibration check solutions with each batch of samples. Components in the samples are identified using retention time and mass spectral criteria. After detection, the individual peaks are integrated and quantified. The wet weight concentrations are converted to a dry weight basis using the moisture content of the sample obtained by gravimetric analysis.

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3. Quality Assurance

A method blank, spiked blanks (BTEX and gasoline), matrix spike (gasoline-spiked onto a soil sample), and a replicate sample are analyzed with each batch of twenty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

METHOD SUMMARY

Total Extractable Hydrocarbons (>C_{10}-C_{32}) in Soil and Sediment

2010/04/15

SOP #: ATL SOP 111

Reference: Atlantic PIRI Guidelines for Laboratories, Version 2, 2006.

1. Scope and Application

This method is designed for the extraction and analysis of petroleum hydrocarbons, including diesel range organics (>C_{10}-C_{21}) and lubricating oils (>C_{21}-C_{32}) in sediments. The reporting limits are as follows: Diesel Range (>C_{10}-C_{21}) – 15 mg/kg; Lubricating Oil Range (>C_{21}-C_{32}) – 15 mg/kg.

2. Summary of Method

A 5 gram portion of wet sediment is weighed out and spiked with two surrogate compounds (isobutylbenzene and n-dotriacontane). These compounds represent a range of volatilities and are used to monitor the efficiency of the sample preparation. The sample is extracted by vigorous shaking with 50:50 (v/v) acetone:hexane. The extract is partitioned with the addition of water to separate the acetone and hexane extract. The extract is then analyzed by capillary column gas chromatography with split/splitless injection and flame ionization detection (GC-FID).

Characterization and quantitation of the sample components are obtained by comparing instrumental responses with those of prepared multi-component standards. Calibration accuracy is verified by analyzing independent reference standards. The day-to-day stability of the calibration is confirmed by analyzing calibration check solutions with each batch of samples. The wet weight concentrations are converted to a dry weight basis using the moisture content of the sample obtained by gravimetric analysis.

3. Quality Assurance

Sample duplicates, process spikes, matrix spikes and method blanks are prepared and analyzed with each batch of 20 samples. Process and matrix spikes are fortified with known concentrations of transformer oil.

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METHOD SUMMARY

Title: Low Level Extractable Hydrocarbons (>C_{10}-C_{32}) in Sediment November 2006

SOP #: ATLSOP 00115/1
Reference: Atlantic RBCA Guidelines for Laboratories, Version 2, January 2006.

1. Scope and Application

This method is designed for the extraction and analysis of >C10 – <C32 petroleum hydrocarbons, including diesel range organics (>C10 - C21) and lubricating oils (>C21 - <C32) in water, seawater, and waste water samples. The reporting limits are as follows: Diesel Range (C_{11}-C_{20}) – 0.25 mg/kg; Lubricating Oil Range (C_{20}-C_{32}) – 0.25 mg/kg.

This method is used in conjunction with SOP #s ATLSOP00117, "Volatile Petroleum Hydrocarbons in Soil" or ATLSOP00119, "Volatile Petroleum Hydrocarbons in Soil by Headspace/GCMS/FID" to quantify Total Petroleum Hydrocarbons (C6 – <C32) in a sample.

2. Summary of Method

A suitable portion of wet sediment is weighed out and spiked with two surrogate compounds (isobutylbenzene and n-dotriacontane). These compounds are used to monitor the efficiency of the sample preparation steps. The sample is extracted by vigorous shaking with 50%/50% (v/v) acetine/hexane sovent mixture. Non-petrogenic compounds are removed using silica gel. The extract is then concentrated and analyzed by capillary column gas chromatography with split/splitless injection and flame ionization detection.

The GC-FID system is calibrated with standards of known concentration. Each standard contains 9 different aromatic and aliphatic compounds which elute in the >C10 – <C32 range. A calibration curve is generated for each carbon range (>C10- C21 and >C21 – <C32) by taking an average response factor of the compounds that elute in each range. Calibration accuracy is verified by analyzing an independent reference standard. The day-to-day stability of the calibration is confirmed by analyzing calibration check standards with each batch of samples. Sample products are identified by comparing each product to a library of reference products.

3. Quality Assurance

Method blanks, spiked blanks (transformer oil), matrix spikes (transformer oil spiked into water samples), and replicate samples are analyzed with each batch of forty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

METHOD SUMMARY

Mercury in Soils, Sediments and Solids

SOP #: ATL SOP-00026

Reference: USEPA Method 245.5.

1. Scope and Application

This method is designed for the digestion and analysis of total mercury in soil, sediment and solid samples as referenced in EPA Method 245.5. The Reporting Detection Limit (RDL) for this procedure is <0.01 mg/kg based on an initial dry weight of 0.3 grams.

2. Summary of Method

The sample is dried at 60°C and sieved. Approximately 0.3 grams is accurately measured for analysis and digested at 95°C with a mixture of sulphuric acid, nitric acid and an excess of potassium permanganate, along with potassium persulphate. Following digestion, excess potassium permanganate is destroyed with hydroxylamine hydrochloride and samples are analyzed for mercury using an automated CVAAS Analyzer. During analysis, the digested samples are mixed with stannous chloride which reduces the mercury to its atomic state. The mercury is sparged from solution using nitrogen gas and the vapour is swept into the absorption cell. The instrument signal (absorbance) at 253.7 nm is proportional to the concentration of mercury in the sample.

3. Quality Assurance

Certified reference materials, method blanks, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of 1 for every 20 samples.

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METHOD SUMMARY

Polycyclic Aromatic Hydrocarbons in Soils and Sediments

SOP #: ATL SOP-000102

Reference: USEPA Method 8270 C - Semivolatile Organic Compounds By Gas Chromatography/Mass Spectrometry, USEPA Method 3540 C - Soxhlet Extraction, and USEPA Method 3630 C - Silica Gel Clean Up

1. Scope and Application

This method is applicable to the determination of polycyclic aromatic hydrocarbons (PAHs) in soils and sediments with a reporting limit of 0.01 mg/kg for regular soils and 0.005 mg/kg for CCME soils. The following compounds are routinely determined:

Analyte	Analyte	Analyte
Naphthalene	Anthracene	Benzo[*j*]fluoranthene
1-Methylnaphthalene	Fluoranthene	Benzo[*a*]pyrene
2-Methylnaphthalene	Pyrene	Perylene
Acenaphthylene	Benz[*a*]anthracene	Indeno[*1,2,3-cd*]pyrene
Acenaphthene	Chrysene	Dibenz[*a,h*]anthracene
Fluorene	Benzo[*b*]fluoranthene	Benzo[*ghi*]perylene
Phenanthrene	Benzo[*k*]fluoranthene	

Other PAHs can be analyzed by this method provided appropriate standards are available.

2. Summary of Method

A representative 5 gram portion of wet soil or sediment is weighed out and spiked with 3 deuterated surrogate PAH compounds. These compounds are used to monitor the efficiency of the sample preparation steps. The sample is extracted for 30 minutes by vigorous shaking with a mixture of 50:50 (v:v) acetone:hexane. The hexane is partitioned from the acetone by the addition of organic free water. If required, interfering

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compounds are removed using a silica gel solid phase extraction (SPE) clean-up procedure. The extract is analyzed by capillary gas chromatography/mass spectrometry (GC/MS) using selected ion monitoring mode.

The GC/MS system is calibrated with PAH standards of known concentration. Calibration curves are prepared by integrating the areas of target ions of the individual PAH peaks obtained during the calibration runs. Calibration accuracy is verified by analyzing an independent reference standard. The day-to-day stability of the calibration is confirmed by analyzing calibration check standards with each batch of samples. The components in the samples are identified using retention time criteria and qualifier ion ratios. After being detected, the individual peaks are integrated and quantified. The wet weight concentrations for each sample are converted to dry weight concentrations using the sample percent moisture value. The percent moisture of each sample is determined separately by gravimetric analysis.

3. Quality Assurance

A method blank, spiked blanks (each individual PAH), matrix spike (each PAH spiked onto a soil sample), and a replicate sample are analyzed with each batch of twenty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

METHOD SUMMARY

Particle Size Distribution of Soils and Sediments

SOP #: ATL SOP-00012

Reference: Methods for Sampling and Analysis of Marine Sediments, Ocean Dumping Report 1, Section 5 – Physical Analysis.

1. Scope and Application

This method is suitable for the measurement of particle size in solid samples ranging from 2 mm to 2 um. Gravel, sand, silt and clay as defined by the Wentworth Method of Classification may also be determined.

2. Summary of Method

A 20 - 30 gram portion of 'as received' sample is sub-aliquotted for analysis. Excess organic matter and carbonates are destroyed by adding hydrogen peroxide and allowing to stand overnight. The sample is dispersed in distilled water and Calgon solution by mechanical shaking. The sand fraction is separated from the silt and clay by wet sieving through a 63 micron mesh size sieve. The particles remaining on the sieve (i.e. the gravel and sand fractions) are dried and then passed through a nest of sieves in order to separate the fractions using a mechanical shaker. Each fraction is determined gravimetrically.

The silt and clay suspension passing through the 63 micron sieve are transferred to a 1000 mL graduated cylinder for pipette analysis. Aliquots are extracted at specific depths and times as defined by a pipetting schedule. These aliquots are transferred to pre-weighed dishes which are placed in a convection oven at 105C and allowed to dry to constant weight. The diameter for each fraction is determined based upon Stoke's Law which relates the settling velocity of spherical particles to their diameter. Gravel, sand, silt and clay fractions are then calculated using the Wentworth method of classification. A graphical interpretation is supplied with the final results.

3. Quality Assurance

Duplicate analysis of samples is performed at a frequency of 5 %.

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Preparation of AVS and SEM in Soils

PURPOSE/PRINCIPLE OF METHOD:

The soil sample is placed in a sample tube section of a micro-distillation stick, which contains a small amount of RODI water. The slurry is acidified and sealed to the micro-distillation stick that has a collection tube section containing sodium hydroxide trapping solution. The micro-distillation stick is placed in a block heater; distillation occurs within the micro-distillation stick that has a hydrophobic membrane between the sample tube and collection tube sections. The distillate is then analyzed to determine the amount of sulfide in soils while the acidified RODI water is analyzed to determine the amount of simultaneously extracted metals. The sulfide may be in the form of S^{2-} , HS- or H2S. Strongly complexed sulfides such as PbS are not determined.

SCOPE:

This method is applicable to soil and sediment samples only

INTERFERENCES:

Soil samples that contain surfactants will cause excessive foaming and plug the distillation membrane thus preventing proper distillation.

SAMPLE HANDLING & PRESERVATION:

Sample Matrix	Sample Container	Hold Time for Sample prep	Storage Conditions	Hold Time before Analysis	Preservation
Soil	Glass jar	1 month	4±2°C.	7 days	None

REFERENCE:

Analytical Method for the Determination of Acid Volatile Sulfide and Selected Simultaneously Extractable Metals in Sediment. Method EPA-821-R-91-100, Revision 1.0, USEPA, December 1991.

METHOD SUMMARY

Total Carbon / Organic Carbon in Soils and Sediments

SOP #: ATL SOP-00044

Reference: Application Bulletin, LECO Corporation Form No. 203-601-224, "Carbon in Soil, Rock, Limestone and Similar Material".

1. Scope and Application

This method is applicable to the analysis of total carbon and organic carbon in soil, sediment samples and compost samples by LECO EC-12 Carbon Analyzer as referenced in Application # 203-601-224 from LECO Equipment Corp. The Reporting Detection Limit (RDL) for this procedure is 0.2 g/kg based on combustion of 0.5g sample.

2. Summary of Method

For Total Carbon analysis, a sample is combusted with oxygen in an induction furnace in the presence of a copper accelerator (and additional accelerators if required). Approximately 97% of the carbon in the sample is oxidized to CO_2 and about 3% combusts to CO, which is catalytically converted to CO_2. Combustion gases are swept into an infra-red detector cell where the CO_2 is measured.

Organic Carbon requires pre-treating the sample in order to remove the inorganic carbon. The sample is digested with hydrochloric acid in order to drive off all carbonates, and then dried prior to the above analysis.

3. Quality Assurance

A minimum of one method blank, one duplicate and one standard reference material is analyzed for each set of samples.

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METHOD SUMMARY

Total Metals in Soil / Sediment Samples

SOP #: ATL SOP-00052

Reference: Based on ISO 14869-1.2001: *Soil Quality - Dissolution for the Determination of Total Elements Content- Part 1: Dissolution with hydrofluoric and perchloric acids.*

1. Scope and Application

This method is applicable to the digestion and analysis of soil and sediment samples for the determination of total trace metals. Lower Reporting Detection Limits (RDLs) or additional parameters may be available upon request.

Analyte	RDL[1]	Analyte	RDL[1]
Aluminum	10	Molybdenum	2
Antimony	2	Nickel	2
Arsenic	2	Phosphorus	100
Barium	5	Potassium	100
Beryllium	2	Rubidium	2
Bismuth	2	Selenium	2
Cadmium	0.15	Sodium	100
Calcium	100	Silver	0.5
Chromium	2	Strontium	5
Cobalt	1	Tellurium	2
Copper	2	Thallium	0.1
Iron	50	Tin	2
Lead	0.5	Uranium	0.1
Lithium	2	Vanadium	2
Magnesium	100	Zinc	5
Manganese	2		

Note 1: Reported in mg/kg air dry weight basis (based on 0.5 g made to a final volume of 50 mL with a x10 dilution prior to analysis).

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2. Summary of Method

A portion of the air-dried sieved (< 2mm) sample is accurately weighed and digested using a mixture of perchloric, nitric and hydrofluoric acids. All samples are analyzed by ICP-MS in accordance with USEPA SW846 Method #6020A. The multi-elemental determination of trace metals by ICP-MS is accomplished by measuring the ions produced when the dissolved sample is introduced into radio frequency inductively coupled plasma. These ions are sorted according to their mass-to-charge ratios and quantified with a discrete dynode electron multiplier detector.

3. Quality Assurance

Certified reference materials, method blanks, blank spikes, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of one for every twenty samples.

METHOD SUMMARY

Weak Acid Extractable Metals in Sediment Samples

SOP #: ATL SOP-00058

Reference: A. Tessier et al, Analytical Chemistry Vol. 51 No. 7, June 1979. *"Sequential Extraction Procedure for the Speciation of Particulate Trace Metals"*

1. Scope and Application

This method is applicable to the extraction and analysis of sediment samples for the determination of weak acid extractable trace metals. Lower Reporting Detection Limits (RDLs) or additional parameters may be available upon request.

Analyte	RDL[1]	Analyte	RDL[1]
Aluminum	10	Lead	0.5
Antimony	2	Manganese	2
Arsenic	2	Molybdenum	2
Barium	5	Nickel	2
Beryllium	2	Selenium	2
Boron	5	Silver	0.5
Cadmium	0.3	Strontium	5
Chromium	2	Thallium	0.1
Cobalt	1	Uranium	0.1
Copper	2	Vanadium	2
Iron	50	Zinc	5

Note 1: Reported in mg/kg air dry weight basis.

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2. Summary of Method

An aliquot of sample is accurately weighed and extracted for some or all of the following fractions (1 – 4):

1. Exchangeable
2. Bound to Carbonates
3. Bound to Iron or Manganese Oxides
4. Bound to Organic Matter

The residual sediment may be digested for total or available metals.
The combined extract or individual extracts are analyzed by ICP-MS in accordance with USEPA SW846 Method #6020A. The multi-elemental determination of trace metals by ICP-MS is accomplished by measuring the ions produced when the dissolved sample is introduced into a radio frequency inductively coupled plasma. These ions are sorted according to their mass-to-charge ratios and quantified with a discrete dynode electron multiplier detector.

3. Quality Assurance

A minimum of one reagent blank and one duplicate sample are prepared and analyzed with every 20 samples.

EPS 1/RM/35 – December 1998
Method Development and Application Section
Environmental Technology Centre
Environment Canada

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.

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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 ≥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 ≥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.

Section 7

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 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

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 ≥90% control survival; and seven of the 32 tests (= 22%) failed to achieve ≥85% control survival. However, 29 of the 32 tests (= 91%) achieved ≥80% control survival (Doe and Jackman, 1998). Given this historical control performance, a minimum mean 10-day survival rate of ≥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 ≥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 ≥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).

RECOMMENDED GUIDELINES FOR CONDUCTING LABORATORY BIOASSAYS ON PUGET SOUND SEDIMENTS

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Revised July 1995

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LIST OF ACRONYMS

ASTM	American Society for Testing and Materials
DMSO	dimethyl sulfoxide
EC_{50}	median effective concentration
Ecology	Washington Department of Ecology
Eh	oxidation potential
EPA	U.S. Environmental Protection Agency
LC_{50}	median lethal concentration
PCB	polychlorinated biphenyl
PSDDA	Puget Sound Dredged Disposal Analysis
PSEP	Puget Sound Estuary Program
PSWQA	Puget Sound Water Quality Authority
PTFE	polytetrafluoroethylene (Teflon®)
QA/QC	quality assurance and quality control
RTG-2	rainbow trout gonad cell
UV	ultraviolet

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INTRODUCTION

This document presents recommended guidelines for conducting the following laboratory sediment bioassays in Puget Sound:

- Amphipod bioassay
- Bivalve larvae bioassay
- Echinoderm embryo bioassay
- Anaphase aberration bioassay
- Microtox bioassay
  - Organic extraction
  - Saline extraction
- Juvenile polychaete bioassay.

The sediment bioassays considered in this document are generally the tests used most frequently by a variety of Puget Sound investigators. Each recommended guideline is based on the results of a workshop and written reviews by representatives from most organizations that fund or conduct environmental studies in Puget Sound (Table 1). The purpose of developing these guidelines is to encourage all Puget Sound investigators conducting monitoring programs, baseline surveys, and intensive investigations to use standardized methods whenever possible. If this goal is achieved, most data collected in the sound will be directly comparable and thereby capable of being integrated into a sound-wide database. Such a database is necessary for developing and maintaining a comprehensive water and sediment quality management program for Puget Sound.

The recommended guidelines for each sediment bioassay describe the use and limitations of the respective toxicity endpoints; the field collection and processing methods; and the laboratory analytical, quality assurance and quality control (QA/QC), and data reporting procedures. In developing the recommended guidelines, it was recognized that the field of sediment bioassays is relatively new and is expanding rapidly. The loose-leaf format of this document will allow modification of the recommended guidelines, if necessary, and inclusion of guidelines for additional sediment bioassays.

Although the following guidelines are recommended for most studies conducted in Puget Sound, departures from these methods may be necessary to meet the special requirements of individual projects. If such departures are made, however, the funding agency or investigator should be aware that the resulting data may not be comparable with most other data of that kind

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and will be identified as such in the Puget Sound database maintained by the Puget Sound Water Quality Authority (PSWQA). In some instances, data collected using different methods may be compared if the methods are inter-calibrated adequately.

Before guidelines for specific bioassays are described, sections are presented on 1) the criteria used to select the tests considered in this document, 2) protocols for field collection of surficial test sediments, and 3) general QA/QC procedures that apply to all sediment bioassays.

BIOASSAY SELECTION

A large number of marine sediment bioassays have been developed and used in recent years (e.g., Swartz et al. 1979, 1985; Chapman et al. 1985; Schiewe et al. 1985; Williams et al. 1986; Long and Buchman 1989; Pastorok and Becker 1990). Many of these tests have been used to evaluate the toxicity of Puget Sound sediments. Nine tests were considered for inclusion in this document based on the following criteria:

- **Sensitivity**—each test has detected biological effects in a variety of sediments
- **Usage**—each test has been used in more than one study in Puget Sound.

Of the nine sediment bioassays selected for consideration, six were identified as suitable for general application in Puget Sound for reasons outlined in Table 2. These six tests are categorized in Table 3 by kind of test and kind of effect measured. The other three tests, which represent promising techniques to be developed further, include the oligochaete respiration test (Chapman et al. 1985), the surf smelt partial life-cycle test (Chapman et al. 1985; Casillas et al. 1989), and the copepod partial life-cycle test (Misitano 1983; Chapman, unpublished data).

For the 1994 revision effort, two additional bioassays were suggested for inclusion in this chapter: solid phase Microtox and a bioaccumulation bioassay. They will be further considered in the next revision effort as addenda to this chapter.

TABLE 2. APPLICABILITY AND RELEVANCE OF SELECTED LABORATORY SEDIMENT BIOASSAYS

Bioassay[a]	*Species*	Primary Endpoint(s)	Comments
Amphipod 10-day test	*Rhepoxynius abronius* *Eohaustorius estuarius* *Ampelisca abdita*	Mortality	Simple test, reproducible, ecologically relevant, applicability verified in numerous studies, extensive usage to date in Puget Sound
Bivalve larvae 48-hour test	*Crassostrea gigas* *Mytilus edulis*	Abnormality Mortality	Simple test, relevance and applicability verified in several independent studies, extensive usage to date in Puget Sound
Echinoderm embryo test	*Strongylocentrotus purpuratus* *S. droebachiensis* *Dendraster excentricus*	Combined Abnormality Mortality	Simple sensitive test, can be conducted year-round by using different echinoderm species, extensive usage to date in Puget Sound
In vitro anaphase aberration tests with rainbow trout gonad cells	*Oncorhyncus mykiss*	Abnormal anaphases	One of the few methods to measure possible genotoxic/cytotoxic effects, minor usage to date in Puget Sound, requires a higher level of expertise than other tests but can be conducted by more than one laboratory in this region
Microtox, bacterial luminescence bioassay	*Photobacterium phosphoreum*	Luminescence	One of the few methods to measure possible effects on bacteria, shows good promise, extensive usage to date in Puget Sound
Juvenile polychaete test	*Neanthes sp.*	Biomass	Simple sublethal test, measures growth (biomass) response and mortality, applicability verified in a series of studies, minor usage to date in Puget Sound

[a] Primary references for bioassays are as follows:

Amphipod test - Swartz et al. (1985); DeWitt et al. (1989); ASTM (1993b)
Bivalve test - Chapman and Morgan (1983)
Echinoderm test - Dinnel and Stober (1985); ASTM (1993a)
Anaphase test - Landolt and Kocan (1984a,b)
Microtox test - Schiewe et al. (1985); Williams et al. (1986)
Polychaete test - Johns et al. (1990).

TABLE 3. CATEGORIZATION OF SELECTED SEDIMENT BIOASSAYS BY KIND OF TEST AND KIND OF EFFECT

	Kind of Effect[a]		
Kind of Test	Lethal	Sublethal	Genotoxic
Solid and liquid phase:			
Amphipod	X	X	
Bivalve larvae	X	X	[b]
Echinoderm embryo	X[c]	X[c]	[b]
Juvenile polychaete	X	X	
Extracts:			
Microtox		X	
Anaphase aberration			X

[a] Only the juvenile polychaete test measures long-term effects (i.e., 20 days, which is a substantial portion of the test organism's life cycle).

[b] Genotoxic endpoints have been measured by others outside the Puget Sound area.

[c] Unlike bivalve larvae, echinoderm larvae rarely disintegrate. While mortality can be calculated, it is more of a reflection of embryos lost in the sediments rather than embryos lost to disintegration. Thus a combined mortality/abnormality endpoint is more appropriate.

FIELD COLLECTION OF SUBTIDAL SURFICIAL SEDIMENTS

This section describes the protocols required to collect an acceptable subtidal surficial sediment sample for analysis by a laboratory sediment bioassay. This subject has generally been neglected in the past and sampling crews have been given relatively wide latitude in deciding how to collect samples. However, because sample collection procedures influence the results of all subsequent laboratory and data analyses, it is critical that samples be collected using acceptable and standardized techniques.

DESIGN OF SAMPLER

In Puget Sound, the most common sampling device for subtidal surficial sediments is the modified van Veen bottom grab. However, various sampling devices (e.g., Smith-McIntyre grab sampler, box corer, Kasten corer, Minisoutar) are also used. The primary criterion for an adequate sampler is that it consistently collects undisturbed samples to the required depth below the sediment surface without contaminating the samples. An additional criterion is that the sampler can be handled properly onboard the survey vessel. An otherwise acceptable sampler may yield inadequate sediment samples if it is too large, heavy, or awkward to be handled properly. It is recommended that the sampler be constructed of stainless steel to avoid metals contamination which might be likely to occur with brass or other construction materials. One potential source of sampling devices is the University of Washington Department of Oceanography.

Collection of undisturbed sediment requires that the sampler:

- Creates a minimal bow wake when descending
- Closes to form a leak-proof seal after the sediment sample is taken
- Prevents sediment washout and excessive sample disturbance when ascending
- Allows easy access to the sample surface.

Most modified van Veen grabs have open upper faces that are fitted with rubber flaps. Upon descent the flaps are forced open to minimize the bow wake, whereas upon ascent the flaps are forced closed to prevent sample washout. Some box corers have solid flaps that are clipped open upon descent and snap shut after the corer is triggered. Although most samplers seal adequately when new, the wear and tear of repeated field use eventually reduces this sealing ability (e.g., through chipped or improperly aligned jaws). A sampler should therefore be properly maintained and monitored constantly for proper operation and minimal sample leakage. If unacceptable leakage occurs or the sampler malfunctions in any manner, the
sampler should be repaired or replaced. If a sampler is to be borrowed or leased for a project, its operation and sealing ability should be evaluated prior to sampling. Also, it is prudent to have a backup sampler onboard the survey vessel if the primary sampler begins leaking during a cruise.

The required penetration depth below the sediment surface is a function of the desired sample depth (see *Penetration Depth*). Generally, it is better to penetrate below the desired sample depth to minimize sample disturbance when the sampling device closes. Penetration depth of most sampling devices varies with sediment character; it is greatest in fine-grained sediments and least in coarse-grained sediments. Sampling devices generally rely upon either gravity or a piston mechanism to penetrate the sediment. In both cases, penetration depth can be modified by adding or removing steel or lead weights from the samplers. Thus, it is optimal to use a sampler that has a means of weight adjustment. If a sampler cannot consistently achieve the desired penetration depth, an alternate device should be used.

The sampler should be brought aboard the vessel with a minimum amount of swinging, to minimize sample disturbance. Once the sampler is secured onboard the survey vessel, it is essential that the surface of the sample be made accessible without substantially disturbing the sample. Most samplers have hinged flaps on their upper face for this purpose. The openings in the upper face of the sampler should be large enough to allow convenient subsampling of the sediment surface. If an opening is too small, the sample may be unduly disturbed as the field member struggles to take a subsample.

PENETRATION DEPTH

For characterizing the toxicity of surficial sediments in Puget Sound, it is recommended that the upper 2 cm of the sediment in a sample be evaluated. The upper 2 cm of sediment is recommended for analysis because that is the sediment horizon in which most infaunal organisms reside and the horizon that is contacted most frequently by epifaunal organisms. When collecting the upper 2 cm of sediment, it is recommended that a minimum penetration depth of 4-5 cm be achieved for each acceptable sample. The portion of sample below the upper 2 cm of sediment can be discarded after the surficial sediment has been collected (unless the study design specifies otherwise).

Although the 2-cm specification is arbitrary, it will ensure that:

- Relatively recent sediments are sampled
- Adequate volumes of sediment can readily be obtained to satisfy the needs of most study objectives
- Data from different studies (historical or ongoing) can be compared validly.

Sampling depths other than the upper 2 cm may be appropriate for specific purposes. For example, when toxicity determinations are made for sediments to be dredged as part of the permitting process, sediments collected from depths as great as several meters may be tested. Additionally, some investigations may want to examine concentration gradients of contaminants

with sediment depth in order characterize the history of some contamination. It should be remembered, however, that if a sampling depth other than the upper 2 cm is used, the results based on the alternative depth may not be equivalent to results based on the upper 2 cm of sediment.

For sampling of reference sediments it may be appropriate to avoid anoxic sediments below the redox potential discontinuity (RPD) horizon. In the Puget Sound Dredged Disposal Analysis program,

reference sediments with high sulfides are suspected of producing performance problems in some bioassays.

OPERATION OF SAMPLER

The sampling device should be attached to the hydrowire using a ball-bearing swivel. The swivel will minimize the twisting forces on the sampler during deployment and ensure that proper contact is made with the bottom. For safety, the hydrowire, swivel, and all shackles should have a load capacity at least 3 times greater than the weight of a full sampler. In addition, screw-pin shackles should have wire through the eye and around one side of the shackle to prevent the pin from rotating.

The sampler should be lowered through the water column at a controlled speed of approximately 1 foot/second. Under no circumstances should the sampler be allowed to "free fall" to the bottom, as this may result in premature triggering, an excessive bow wake, or improper orientation upon contact with the bottom. The sampler should contact the bottom gently, and only its weight or piston mechanism should be used to force it into the sediment.

After the sediment sample is taken, the sampler should be raised very slowly off the bottom and then retrieved at a controlled speed of approximately 1 foot/second (0.3 meters/second). Before the sampler breaks the water surface, the survey vessel should head into the waves (if present) to minimize vessel rolling. This maneuver will minimize swinging of the sampler after it breaks the water surface. If excessive swinging occurs or if the sampler strikes the vessel during retrieval, extra attention should be paid to evaluating sample disturbance when judging sample acceptability.

The sampler should be secured immediately after it is brought onboard the survey vessel. If the sampler tips or slides around before being secured, extra attention should be paid to evaluating sample disturbance.

SAMPLE ACCEPTABILITY CRITERIA

After the sampler is secured on deck, the sediment sample should be inspected carefully before being accepted. The following acceptability criteria should be satisfied:

- The sampler is not overfilled with sample such that the sediment surface is pressed against the top of the sampler
- Overlying water is present (indicates minimal leakage)
- The overlying water is not excessively turbid (indicates minimal sample disturbance)
- The sediment surface appears to be relatively undisturbed (i.e., lack of channeling or sample washout)
- The desired penetration depth is achieved (e.g., 4-5 cm for a 2-cm-deep surficial sample).

If a sample does not meet all of these criteria, it should be rejected and discarded away from the sampling station.

SAMPLE COLLECTION

After a sample is judged acceptable, the following observations should be entered on the field log sheet:

- Date and time
- Station location at the time of bottom contact (see recommended protocol for station positioning)
- Station depth (relative to MLLW)
- Gross characteristics of the surficial sediment
  - Texture
  - Color
  - Biological structures (e.g., shells, tubes, macrophytes)
  - Presence of debris (e.g., wood chips, wood fibers, human artifacts)
  - Presence of oily sheen
  - Obvious odor (e.g., hydrogen sulfide, oil, creosote)
- Gross characteristics of the vertical profile (determined after the surficial sediments have been collected)
  - Vertical changes in sediment characteristics
  - Presence and depth of any apparent redox potential discontinuity layer
- Penetration depth.

Before subsamples of the surficial sediments are taken, the overlying water must be removed. The preferred method of removing this water is by slowly siphoning it off near one side of the sampler. Methods such as decanting the water or slightly opening the sampler to let the water flow out are not recommended, as they may result in unacceptable disturbance or loss of fine-grained surficial sediment and organic matter.

Once the overlying water has been removed, the surficial sediment can be subsampled. It is recommended that subsamples be taken using a flat scoop. This device will allow a relatively large subsample to be taken accurately to a depth of 2 cm. Coring devices are not recommended because they usually collect inadequate amounts of surficial sediment, and therefore require repeated extractions to obtain a sufficient volume of material for analysis of conventional sediment variables. A curved scoop is not recommended because it does not sample a uniform depth. Because accurate and consistent subsampling requires practice, it is advisable that an experienced person perform this task.

Finally, sample contamination during collection must be avoided. All sampling equipment (e.g., scoops, containers) should be made of noncontaminating material, and should be cleaned appropriately before use. It is recommended that all objects coming in contact with the sample be made of glass, stainless steel, or PTFE (i.e., polytetrafluoroethylene; e.g., Teflon®). To avoid contamination, all sampling equipment should be cleaned in sequence with site seawater, a detergent scrub and rinse, pesticide-grade acetone, and pesticide-grade methanol or hexane prior to initial use and between use for each station. The methanol or hexane should be allowed to evaporate prior to using the equipment. If metals samples are being taken, equipment should also be cleaned with dilute acid (10% HNO_3) and rinsed, followed by the pesticide-grade acetone steps. Where possible, innocuous or biodegradable sampling gear cleaning and decontaminating agents should be used.

SAMPLE HOMOGENIZATION

Sediment from single samples or composites of multiple collections or composites of multiple samples should be homogenized prior to collecting a subsample for bioassay analyses. Compositing and homogenization can be accomplished by transferring sediment to a clean glass or stainless steel bowl and thoroughly homogenizing by stirring with stainless steel spoons or spatulas until textural and color homogeneity are achieved. The contents of the bowl should be continuously homogenized as subsamples are taken, to prevent potential settlement of larger particles. In addition, unrepresentative material (e.g., stones, wood chips, seagrass) should be removed at the discretion of the chief scientist and noted in the field logbook. The bowl and all utensils should be cleaned in sequence with site seawater, a detergent scrub and rinse, pesticide-grade acetone, and pesticide-grade methanol or hexane between composites, and kept covered with aluminum foil to prevent airborne or other contamination. The methanol or hexane should be allowed to evaporate prior to using the bowl and utensils. If metals samples are being taken, equipment should also be cleaned with dilute acid (10% HNO_3) and rinsed, followed by the pesticide-grade acetone steps. If total sulfides are to be measured, a subsample should be taken immediately. Zinc acetate should be used as a fixative following the recommended protocol for measuring conventional sediment variables.

CONCURRENT COLLECTION OF SEDIMENT CHEMISTRY AND BIOASSAY SAMPLES

If sediment chemistry samples are being collected concurrently with sediment bioassay samples, they should be collected from the same homogenized sediment sample. Specifically, individual samples or composites of several samples should be mixed before aliquots are removed for sediment chemistry and sediment bioassay determinations. Sample homogenization and removal of bioassay aliquots should be conducted so that chemical aliquots are not contaminated in the process. The protocols for removing aliquots for analyses of organic compounds and metals should be consulted in the appropriate Puget Sound guidelines documents (PSEP 1989a,b) to ensure that removal procedures for bioassay

aliquots are compatible with those recommended for chemical aliquots.

REPLICATION OF BIOASSAYS

The guidelines for all of the sediment bioassays included in this document recommend that all field-collected sediments be homogenized before subsamples are removed for bioassay and chemical analyses. This technique is the one used for most studies in Puget Sound, and it ensures that the bioassay and chemical results are related as closely as possible. The replicate analyses that are subsequently conducted on the bioassay subsample assess the variability encountered in laboratory testing, rather than the variability of sediment toxicity that exists in the field. To assess field variability, an alternate sampling design could be specified that requires each bioassay replicate to be run on a separate replicate grab sample from each station. The primary drawback to this technique is that the single set of chemical concentrations usually measured at each station (i.e., primarily because of cost constraints) would not relate directly to the sediment toxicity measured in each replicate grab sample. This lack of a direct relationship between bioassay and chemical results can sometimes make data interpretation difficult for individual replicate samples. However, the mean bioassay response could be compared directly with chemical concentrations if the chemical measurements are made on a composite of equal amounts of sediment subsampled from each of the replicate samples used for bioassay analysis.

GENERAL QUALITY ASSURANCE/QUALITY CONTROL GUIDELINES

The following general QA/QC procedures apply to all sediment bioassays. QA/QC procedures specific to each of the individual bioassays addressed in this document, and that differ from the following generic guidelines, are incorporated into individual bioassay guidelines.

NEGATIVE CONTROLS

All bioassays must be conducted using well-established negative (clean) controls. Such controls are clean, nontoxic seawater and/or sediment samples taken from outside each study area. For every test series with a particular organism, one bioassay test chamber or series of chambers must contain clean, material (i.e., seawater and/or sediment). In most cases, a complete batch of bioassay analyses must be repeated if more than a specified percentage of the corresponding control animals exhibits the response of interest.

POSITIVE CONTROLS

All bioassays shall be conducted using well-established positive (toxic) controls. These controls involve the use of reference toxicants such as cadmium chloride, silver chloride, phenol, and sodium lauryl sulfate. Use of sodium pentachlorophenate as a reference toxicant should be avoided because of the potential for dioxin contamination and the difficulty encountered in disposing of this chemical. Reference toxicants are used to provide insight into mortalities or increased sensitivity that may occur as a result of disease, changes in tolerance/sensitivity, or loading density. Reference toxicants can also provide insight into nonlethal effects that occur due to acclimation, insensitivity, or stress tolerance developed during handling and acclimation. Also, the use of reference toxicants facilitates comparisons of test results among different studies. Accordingly, concurrent bioassays using a reference toxicant should be implemented for each test series. Labs are advised to investigate proper disposal of reference toxicant in their area, e.g., acceptability for disposal to sanitary sewer systems. Labs should maintain documentation on discharge practices for test solutions, sediments and culture water, including disinfection of discharged water. Finally, it is common practice for labs to establish an internal acceptance standard through control charts (for example, plus or minus two standard deviations on the EC_{50} or LC_{50}).

TEST ORGANISMS

Only healthy organisms of similar size and life history stage should be used in bioassays. Taxonomic identifications of bioassay organisms must be confirmed by a qualified taxonomist. It is recommended that a type specimen be established, with at least annual identification of organisms and maintenance of voucher specimens for each project. Labs should maintain documentation on the source of test organisms and the disposal of test organisms. Some test organisms (for example *Neanthes arenaceodentata* and *Ampelisca abdita*) are not native to Washington and must be managed in compliance with Washington State Department of Fisheries and Wildlife (WDFW) regulations regarding aquatic disease control and the importation of exotic species (Chapter 220-77 WAC, Aquatic Disease Control, and Chapter 232-12-017 WAC, Deleterious Exotic Wildlife). Also, labs are advised to have and maintain the proper WDFW permits for collecting Puget Sound marine organisms.

REFERENCE TEST SAMPLES

Laboratory negative control sediments generally are those from which infaunal test animals (e.g., amphipods) were collected. As such, physical and chemical sediment characteristics (e.g., interstitial water salinity, grain size, organic content) may be very different from those of the test sediments. Where this is the case, one or more reference sediments should be added to the test series. Reference sediments should be collected from an area documented to be free from chemical contamination and should represent the range of important natural physical and chemical characteristics of the test sediments (see section titled *Puget Sound Reference Areas*). Such a sample, if it is in fact essentially free of contamination, can provide data that can be used to separate toxicant effects from unrelated effects such as those of sediment grain size. Additionally, to further ensure the integrity of reference test samples, investigators should use experienced sampling personnel and sample from the biologically active zone, avoiding anoxic sediments below the RPD horizon. The wet-sieving method (PTI, 1991; PSDDA, 1990) should be used to match reference sediments with test sediments.

SEDIMENT HOLDING TIME

At the present time specific holding times for sediment samples used for bioassay testing have not been thoroughly evaluated. The following suggestions are recommended on an interim basis. As additional data are generated, these holding times for bioassay samples will be re-evaluated. Each study (and testing of reference and control sediments) should report the holding times with the study results.

Fresh sediment to be used in the amphipod, bivalve larvae, echinoderm embryo, juvenile polychaete, and Microtox (saline extract) bioassays should be stored in the dark at 4°C for as brief a time as possible after field collection (ideally no more than 2 week). Sediments to be used in the anaphase aberration and Microtox (organic extract) tests should be frozen at -20°C within 8 hours of collection, stored, and extracted with organic solvent within 6 months after field collection.

Although it is largely unknown whether sediment toxicity changes substantially between field collection and laboratory analysis following a holding time of 2 weeks at 4°C, the 2-week holding time is considered the minimum holding time that can be routinely achieved by most studies conducted in Puget Sound. The minimum 2-week holding time is therefore based partly on logistical considerations.

Maximum holding times are important to programs that rely on tiered testing where chemical analyses are conducted prior to toxicity testing. This tiered approach is used by the Puget Sound Dredged Disposal Analysis (PSDDA) program for evaluating dredged sediments for unconfined open-water disposal in Puget Sound. Under the PSDDA program, sediments are stored in the dark in a nitrogen atmosphere at 4°C for no longer than 8 weeks. The PSDDA program has established an 8-week maximum based on Tatem et al. (1991) and Becker and Ginn (1990). Because the results of recent studies evaluating the effects of sediment holding time on sediment toxicity have been variable, it is prudent to store sediments for as short a time as possible after field collection. If there are no other compelling reasons (such as the tiered testing schedule under PSDDA), a maximum holding time of 2 weeks is recommended for Puget Sound, based on the best professional judgment of regional investigators and on logistical constraints.

At the national level, while the American Society for Testing and Materials recommends a maximum holding time of 2 weeks for both marine sediment (ASTM 1990) and freshwater sediment (ASTM in press) prior to toxicity testing, national guidance provided by the joint EPA/Corps Ocean Testing Manual (1991) and Inland Testing Manual (draft 1994) allow longer sediment holding times similar to PSDDA.

Regardless of the holding time used for a study, it is essential that the holding time be reported along with the study results.

BLIND TESTING

For regulatory purposes, blind and random testing is required and all sediment treatment containers should be randomized and testing should be conducted without laboratory personnel knowing sample identities. Replicates of each treatment should be assigned a code number during testing and randomized in the test sequence.

For some research investigations it is recognized that blind testing may not be required.

MAINTENANCE/MEASUREMENT OF WATER QUALITY

Bioassays involving exposure of organisms in aqueous media require that the media be uncontaminated, and that proper water quality conditions be maintained to ensure the survival of the organisms and to ensure that undue stress is not exerted on the organisms unrelated to the test sediments. At a minimum, the following variables must be measured at the beginning and terminations of testing: salinity, dissolved oxygen, pH, and temperature. Other conventional water quality variables, such as sulfides and ammonia, may also influence the results of sediment toxicity testing and should be measured as routine water quality parameters at the beginning and termination of testing. For bioassay tests lasting several days, it is recommended that the following variables be measured on a periodic basis and recorded: salinity, dissolved oxygen, pH, and temperature.

EQUIPMENT CLEANING PROCEDURES

It is essential that field and laboratory equipment be properly cleaned before sediments are collected or bioassays are conducted. Proper cleaning ensures that bioassay responses are related to the contaminants present in the test sediments and not to contaminants present on improperly cleaned equipment. The following cleaning procedures are recommended for all bioassays:

- Sediment collection equipment (e.g., corers, grab samplers, homogenization bowls, and utensils):

  - Wash with laboratory detergent (non-phosphate)
  - Rinse with 10% HNO_3 followed by distilled water
  - Rinse with pesticide-grade acetone
  - Rinse with pesticide-grade methanol or hexane

- Laboratory equipment (e.g., sample containers, bioassay chambers, and utensils):

  - Wash with laboratory detergent
  - Rinse with tap water
  - Rinse with 10-percent reagent-grade hydrochloric acid
  - Rinse 5 times with deionized water
  - Rinse with pesticide-grade acetone
  - Rinse with pesticide-grade methanol or hexane
  - Allow residual methanol or hexane to evaporate

If alternate cleaning techniques are used, it should first be demonstrated that they are as effective as the above techniques, and they shall be documented as a standard operating procedure and kept with the study records.

STANDARD LABORATORY PROCEDURES

Standard laboratory procedures must be followed in all testing. These include proper documentation, proper cleaning, avoidance of contamination, and maintenance of appropriate test conditions. All unusual observations or deviations from established procedures must be recorded and reported.

PUGET SOUND REFERENCE AREAS

Performance standards for reference areas are needed to provide an objective and consistent basis for assessing contaminated sediments in various environments. Performance standards are criteria for suitable reference conditions defined in terms of habitat characteristics (e.g., sediment grain size, salinity, chemical concentrations) and biological properties (e.g., bioassay responses). The Washington Department of Ecology has developed interim performance standards for subtidal sediments in Puget Sound reference areas (Pastorok et al. 1989). Development of reference area performance standards for intertidal areas or for specific biogeographic regions (including low-salinity habitats) requires further data collection. Where quantitative performance standards could not be developed for certain variables, general objectives for locating reference area stations were provided to guide technical judgments regarding the acceptability of reference sites on a case-by-case basis. The recommended performance standards modified from Pastorok et al. (1989) are summarized below.

Reference areas used in evaluating sediments should be relatively clean (i.e., they should be consistent with the performance standards being developed by Ecology). Reference areas should not be located immediately adjacent to contaminated sites (e.g., within the central basin, within the East Passage, or within an urbanized embayment such as Elliott Bay or Commencement Bay). Pastorok et al. (1989) suggested Sequim Bay, Samish Bay, Dabob Bay, and Carr Inlet as the primary reference areas for Puget Sound based on available data. Reference areas used by other investigators of sediment conditions have included Carr Inlet (Tetra Tech 1985); Samish Bay, Dabob Bay, and Sequim Bay (Battelle 1986); Case Inlet (Malins et al. 1980; Battelle 1986); Port Madison (Malins et al. 1980); and Port Susan (Malins et al. 1982; PTI and Tetra Tech 1988a,b). Recent work suggests that other locations (e.g. in Grays Harbor and Willapa Bay) may make suitable reference areas (EPA, 1993). Additional information on specific reference areas may become available as Ecology or other agencies continue to develop performance standards. Sampling stations in reference areas should be located to achieve the following general objectives.

SEDIMENT CHEMISTRY

- Concentrations of all chemicals measured in sediments should be representative of uncontaminated areas of Puget Sound that are remote from significant sources of contaminants.

- Concentrations of all chemicals analyzed in sediments should be lower than available performance standards for Puget Sound reference areas. If such standards are not available for specific compounds, the concentrations of those compounds should be less than the state sediment quality standards.

SEDIMENT BIOASSAYS

- The bioassay response measured for sediments from each reference station should be less than the mean response that can be consistently discriminated as toxic in statistical comparisons (a=0.05) with control sediments (amphipod and juvenile polychaete bioassays), control seawater (bivalve larvae and echinoderm embryo bioassays), or solvent (anaphase aberration and Microtox bioassays).

- The physical and chemical characteristics (e.g., grain size, organic content, interstitial salinity) of reference sediments should match the corresponding characteristics corresponding to those of the test sediments as closely as possible. In some cases it may be appropriate to use the wet-sieving technique (PTI, 1991; PSDDA, 1990) to match reference sample grain sizes to test sediment.

- Quantitative performance standards for sediment bioassays from reference areas are currently being developed by Ecology.

GENERAL HABITAT

- Existing or planned development (e.g., major shoreline or water projects) should be minimal in the vicinity of reference sites

- A reference area should be accessible for sampling by routine techniques.

AMPHIPOD SEDIMENT BIOASSAY

USE AND LIMITATIONS

The amphipod (*Rhepoxynius abronius*, *Ampelisca abdita* or *Eohaustorius estuarius*) sediment bioassay is used to characterize the toxicity of marine or estuarine sediments. This bioassay may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations. Mortality is the primary endpoint in the amphipod bioassay. Sublethal endpoints, such as emergence of amphipods from the sediment during the exposure period and failure to rebury in sediment at the end of the exposure period, may also be used to assess sediment toxicity. Total effective mortality, a combined endpoint representing the sum of percent mortality and percent nonreburial, has also been used in this bioassay for *R. abronius* or *E. estuarius*. The basis for the combined endpoint is the assumption that individuals that fail to rebury in sediments at the end of the exposure period would die in nature as a result of predation.

The following constraints apply:

- For the *R. abronius* bioassay, an interstitial water salinity of ≥25 ppt is necessary to ensure that there are no salinity effects. In general, adjustment of interstitial water salinity should not be attempted because of potential effects of adjustment on toxicological properties of the sediment. However, for dredged material that will be disposed of in the marine environment (where *in situ* sediments have interstitial water salinities >25 ppt), salinity adjustment may be desirable. If interstitial water salinities of dredged material are between 15 and 24 ppt, they may be adjusted upward for use in the *R. abronius* bioassay. For other testing purposes, use of the *E. estuarius* or *A. abdita* bioassay is preferred for sediments with salinities <25 ppt.

- Grain size may have an effect on *R. abronius* or *E. estuarius* at extremes of fine and coarse material (DeWitt et al. 1988, 1989). If the clay and fines content of the test sediments exceeds 20 percent or the gravel content exceeds 35 percent, controls for the effects of particle size distribution (i.e., a reference area sediment similar in grain size to the test sediment) are recommended for interpretation of toxicity test results when using *R. abronius* or *E. estuarius*. At the coarse end of the spectrum (greater than 20 percent gravel), a grain size reference sediment is also recommended for *Ampelisca*.

- As in all bioassays using natural populations, there is a possibility that relative sensitivity of the amphipods will vary with season or other factors. Accordingly, a positive control is recommended. This should comprise a 96-hour LC_{50} measurement with a reference toxicant (e.g., cadmium chloride) conducted in the absence of sediment. The salinity and temperature of the dilution water should be equivalent to the values specified for sediment testing (i.e., 28 ppt and 15°C, respectively).

- Identification of *R. abronius*, *A. abdita* and *E. estuarius* must be confirmed by a qualified taxonomist prior to initiation of the bioassay, and representative specimens should be preserved and archived for future reference.

- Predators generally are not a problem in the bioassay, but potential problems can be avoided by observation and predator removal (if necessary).

The *R. abronius* bioassay is appropriate for sediments with interstitial water salinity of ≥25 ppt. The *E. estuarius* bioassay is appropriate for sediments with interstitial water salinity of 2-28 ppt. *A. abdita* is appropriate for sediments which have greater than 60 percent clay and silt (fines) and with interstitial water salinity < 25 ppt.

Recommended Use Matrix	Sediment fines < 60%	Sediment fines ≥ 60%
Salinity ≥ 25 ppt	*R. abronius*	*A. abdita*
Salinity < 25 ppt	*E. estuarius*	*A. abdita*

These guidelines have been adapted from Swartz et al. (1985) for *R. abronius* and DeWitt et al. (1989) for *E. estuarius*. A standard guide for conducting 10-day static sediment toxicity tests for marine and estuarine amphipods has been developed by ASTM (1990).

FIELD PROCEDURES

Collection

Test Animals—Both *R. abronius* and *E. estuarius* can be collected using benthic grabs (e.g., van Veen, Smith-McIntyre) or small dredges. *E. estuarius* can also be collected by shovel at low tide. *A. abdita* can be collected by grab or by shovel in shallow areas. *A. abdita* is an east coast species which has also been introduced into San Francisco Bay. If a dredge is used, a short haul (10 meters) will minimize potential damage to the animals during collection. *R. abronius* inhabits fine sands from the low intertidal zone to a maximum depth of 60 meters. *E. estuarius* is generally found in intertidal estuarine sediments from +0.5 meters to +2.0 meters above mean lower low water (MLLW). *A. abdita* is found mainly in fine sediments from the low intertidal to 60 m. Approximately one-third more animals than are required for the bioassay are collected. Surface and bottom seawater salinity and temperature are measured at the collection site. Sediment temperature is recorded from the first and last dredge sample. It is recommended that bioassays be conducted within 10 days of amphipod collection.

Sediment—Control, reference, and test sediments should be stored in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 0.25 liters for each bioassay chamber is recommended for each kind of sediment. Because five replicate tests are conducted for each field sample, a minimum sediment sample size of 1.25 liters is recommended for each station. As a general advisory, twice the volume of sediment needed should be collected, however, the actual amount could be project-specific.

Processing

Test Animals—Contents of the dredge or grab sampler are gently washed into a container using seawater of similar temperature and salinity to that at the collection depth. Samples that show evidence of contamination (e.g., oil sheen) are rejected. *R. abronius* and *E. estuarius* will typically bury in the sediment and if necessary can be held in the containers for several hours (at the temperature of the collection depth) prior to sieving. It is preferable to minimize the delay between collection and sieving. Screens for sieving sediments should be either stainless steel or Nitex® type plastic to avoid sediment contamination with metals. To avoid handling stress, each dredge sample is placed in a separate container. Amphipods are maintained and transported in clean coolers, should be held in sediment during transport to the laboratory, and should be kept at or below the collection site temperature. During a long transport, aeration may be required. *Ampelisca* are not as easily removed from the sediment and are sometimes transported to the lab in the tube mat and sieved there.

Sediment—Control, reference, and test sediments should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The laboratory procedures for *R. abronius* and *E. estuarius* are those described by Swartz et al. (1985) and DeWitt et al. (1989) with the following changes incorporated:

- The salinity of the overlying water is adjusted to 28 ppt for *R. abronius*
- A 1.0-mm screen is used to sieve out amphipods prior to initiation of testing
- Holding time for amphipods is standardized to between 2 and 10 days
- Additional details are provided concerning maintenance and transportation of amphipods, confirmation of taxonomic identifications, and freeing of amphipods trapped by water surface tension during testing
- A specific procedure for adjustment of interstitial water salinity for testing dredged material is included.

The laboratory procedures for *A. abdita* are those described by the ASTM (1993b). The following points should be observed when using *A. abdita*:

- *Ampelisca* are fed during the laboratory holding period before the test
- A 0.5-mm screen is used to sieve out amphipods prior to initiation of testing

- The *Ampelisca* test is run at 20°C instead of 15°C
- Holding time for amphipods is standardized to between 2 and 10 days
- Immature amphipods are used in the *Ampelisca* test because if mature male Ampelisca are used, they will likely die of senescence during the 10-day test
- There is no reburial endpoint in the *Ampelisca* test. *Ampelisca* is not indigenous to the West Coast (except for an introduced population in San Francisco Bay), therefore special care must be taken by testing laboratories not to release the animals into the environment.

Test Animals

Sieving (*R. abronius* and *E. estuarius*)—A 1.0-mm sieve is used to remove the amphipods from sediment and mature amphipods (3.0–5.0 mm total length) are used in the sediment bioassay. Gentle sieving is essential to reduce handling stress. The sieve is placed in a large tub filled with seawater at ambient salinity and temperature for the collection site sediments. The entire contents of each holding container, including water, are washed through the sieve using seawater pumped at low pressure through a fan spray nozzle. The sieve can be shaken gently, but the bottom of the screen must be beneath the water surface at all times. Material retained on the screen is washed into buckets for sorting. Large pieces of detritus and obvious predators are discarded. If there is a delay of more than 1 hour before sorting begins, the buckets should contain enough sieved sediment to allow the amphipods to bury. The buckets must be kept at or below collection site temperature. Aeration may be necessary.

Sieving (*A. abdita*)—A 2-mm mesh sieve nesting over a 0.5-mm mesh sieve is used for rinsing the sediment containing the amphipods with a forceful stream of seawater at the collection temperature and salinity. The rinsing should break up the sediment material and also force most of the amphipods out of their tubes. An additional 1-mm mesh sieve may optionally be used to sort the animals by size. Material retained on the 0.5-mm sieve is vigorously shaken and swirled so the fine sediments pass through and the amphipods are separated from tubes, sediment, and detrital material. The sieve is then lifted from the water, allowed to drain, and slowly lowered into a shallow tray of seawater so that the *Ampelisca* will be caught on the water's surface tension and can be collected with a fine mesh dip net.

Sorting—Animals should be presorted. As an example, an aliquot of detritus or sediment containing amphipods is placed in a sorting tray. Healthy, active animals are removed with a bulb pipette (5-mm opening) and placed in 10-cm-diameter finger bowls filled with seawater of appropriate salinity and a 2-cm-deep layer of 0.5-mm sieved collection site sediment. Twenty amphipods are held in each bowl and enough bowls are prepared to provide at least one-third more specimens than are required for the bioassay. Seawater temperature during sorting of amphipods must not exceed 18°C (except for *Ampelisca*). *Ampelisca* is routinely tested at 20°C, and for comparison with other *Ampelisca* test results, 20°C is recommended. Filled finger bowls are submerged in holding tanks supplied with flowing water or aeration where temperature and salinity approximate bioassay conditions. If temperature and salinity adjustments are necessary, they should be made gradually. Healthy

amphipods will remain in the finger bowl sediment and can be retrieved easily when the bioassay is set up. *A. abdita* may leave their tubes and swim. Therefore a screened overflow should be used if this species is held in flowing seawater. Amphipods should be acclimated to laboratory conditions for a minimum of 2 days and a maximum of 10 days before testing.

Control Sediment

R. abronius and *A. estuarius* typically inhabit well-sorted, fine sand. Suggestions for sieving and settling may have to be adjusted for other sediment types. *A. abdita* is a tube-dwelling amphipod found mainly in protected areas and is often abundant in sediments with a high organic content. It generally inhabits sediments from fine sand to mud and silt without shell, although it can also be found in relatively coarser sediments with a sizeable fine component.

Approximately 0.25 liters of control sediment should be collected for each bioassay chamber. This sediment is sieved twice: first, to remove the test species and other macrobenthos and second, to adjust interstitial water salinity. The entire contents of one or more sediment samples, including water and suspended particulate matter, are sieved through a 0.5-mm screen without allowing overflow from the container. After the first sieving, the sediment is allowed to settle for at least 4 hours (preferably 12-16 hours). Overlying water is then decanted and the sediment resieved through a 0.5-mm screen into water of the bioassay salinity (28 ppt for *R. abronius* and *A. abdita*, ambient salinity for *E. estuarius*). Investigators should report methods used to determine ambient salinity in cases where the interstitial salinity of the test sediments varies over a range of salinities. Again, the sediment is allowed to settle for at least 4 hours (preferably 12-16 hours), overlying water is decanted, and the control sediment is held at 4°C until the bioassay chambers are prepared.

Test and Reference Area Sediment

Approximately 0.25 liters of test sediment should be collected for each bioassay chamber. Test sediments should not be wet-sieved, but if large predators or other large organisms are present, they can be removed using forceps or by pressing the sediment through a 2.0-mm screen. The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. *R. abronius* may be adversely affected by salinity stress if the interstitial water salinity is below 25 ppt. For estuarine dredged material designated for disposal in the marine environment and for other sediments, interstitial water salinities below 25 ppt may require adjustment upwards to control for false positives. While both *R. abronius* and *E. estuarius* are tolerant of a range of sediment grain size, particle size may affect test results. For these two species, controling for the effects of particle size distribution with reference sediments is recommended if the silt and clay content exceeds 20 percent or the gravel content exceeds 35 percent. For *A. abdita*, which can be found in relatively coarser sediments with a sizeable fine component, controling for the effect of particle size distribution with reference sediments is recommended if the gravel content exceeds 35 percent. (See applicable constraints, p. 20.)

The *R. abronius* test requires a minimum water column salinity regime of 28 ppt. When the interstitial (i.e., pore water) salinity is below 25 ppt, it must be raised if this test is to be used. The following procedure is recommended. The interstitial salinity of the sediments is determined (e.g., by refractometer using interstitial water collected by centrifugation) and the sediments are placed in the

bioassay chambers with overlying water of a salinity calculated to raise interstitial salinities to a minimum of 25 ppt. The sediments are then carefully and slowly stirred by hand with a clean glass rod for 1 minute, and allowed to settle for at least 4 hours (preferably 12-16 hours). The majority (approximately 75 percent) of the overlying water is then carefully decanted and the interstitial salinity in each chamber confirmed prior to bioassay initiation. The decant water can be retained, salinity adjusted if necessary, and used as the overlying water in the bioassay. Alternatively, fresh seawater with the appropriate salinity can be used as the overlying water. Sediments are slowly mixed with a glass rod after adding the decant water. When the bioassays are terminated, the interstitial salinities are reconfirmed (e.g., by refractometer using interstitial water collected by centrifugation).

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt for the *R. abronius* and *A. abdita* tests and at ambient interstitial salinity for the sediment collection site for the *E. estuarius* test. Temperature of seawater used in bioassays of either *R. abronius* or *E. estuarius* is maintained at 15 ± 1°C. A temperature of 20°C is recommended for *A. abdita.* The bioassay seawater must be uncontaminated, which may necessitate collection of seawater at the amphipod collection site. Seawater in which algal blooms have occurred should not be used. Natural and reconstituted seawater should be held at ≤15°C for no longer than 2 days before inoculation. The quantity of seawater required is dependent on sieving and holding needs and on the number of bioassay chambers.

Bioassay seawater is passed through a filter with 0.45-µm pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salts, brine prepared by freezing or reagent grade chemicals (ASTM 1989). Seawater is prepared within 2 days of the bioassay and stored in covered, clean containers at the bioassay temperature.

Facilities and Equipment

The bioassay chamber is a standard 1-liter glass beaker (10-cm internal diameter) covered with an 11.4-cm diameter glass watchglass. The beakers are placed in a shallow water bath or temperature-controlled room with overhead aeration source. Aeration to each beaker is provided through a 1-mL glass pipette that extends between the beaker spout and watchglass to a depth not closer than 2 cm from the sediment surface. Air is bubbled into the beakers at a rate that does not disturb the sediment. The bioassay temperature is maintained by either the water bath or room temperature control.

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. Large plastic containers and plastic sieves used for preparation and storage of sediment and seawater are preconditioned initially by soaking for 24 hours in seawater and rinsed after each use with clean seawater. They are used only for bioassays and stored in a clean room. Sieves and containers used to collect and store amphipods, seawater, and control sediment are kept separate from those used for test sediment.

Bioassay Procedure

The day before the bioassay is initiated, approximately 175 mL of test sediment are placed in the bottom of the 1-liter bioassay chamber to create a 2-cm-deep layer. Five replicate tests are conducted for each field sample. Chambers are filled to 750 mL with seawater at 28 ppt for the *R. abronius* and *A. abdita* tests or ambient interstitial salinity for the *E. estuarius* test, covered with a watchglass, and placed in a water bath (15°C for *R. abronius* and *E. estuarius*, 20°C for *A. abdita*). Constant illumination is provided by overhead lights. Water in the chambers is aerated without disturbing the sediment surface. The system is allowed to equilibrate overnight before the amphipods are added. When the test is initiated, 20 amphipods are placed in each chamber and the seawater level is brought up to 950 mL. The bioassay is terminated after 10 days of exposure.

The primary endpoint is mortality after 10 days exposure to test or control sediment. The secondary endpoints that also can be measured are daily emergence of amphipods from sediment and for *R. abronius* and *E. estuarius* failure to rebury in sediment at the end of the exposure period. It is recommended that the number of amphipods reburying and the number removed from the test be recorded. This information may provide an indication of the viability of the test stock of amphipods.

Initiation—The day before the bioassay is initiated, each test sediment sample is homogenized and an aliquot of 175 mL (sufficient to make approximately a 2-cm-deep layer) is added to a bioassay chamber. For replicate bioassay samples, the volume of sediment necessary to place 175 mL in the first chamber is added to the other replicates. The same procedure applies to control and reference sediment. Treatments are randomly assigned to prenumbered bioassay chamber.

The sediment aliquot in the chamber can be settled by smoothing with a spoon, and bubbles can be removed by tapping the beaker against the palm of the hand. A disk (attached by a string for removal) is placed on the sediment surface. This minimizes sediment disruption as bioassay seawater is added up to the 750-mL mark on the chambers. This disk is removed and rinsed in bioassay water between chambers and changed between treatments. The beakers are covered with watchglasses, put into the water bath of the appropriate temperature or the temperature-controlled room, and aerated. The chambers are allowed to equilibrate overnight to bioassay conditions. Normal room lighting is maintained continuously during the bioassay. If the experimental design requires monitoring of sediment chemistry [e.g., metals, total volatile solids, oxidation potential (Eh)], additional chambers must be set up for this purpose. Monitoring the quality of seawater overlying the sediment can be accomplished in the bioassay chambers without disturbing the sediment. Temperature is recorded from a thermometer maintained in a separate chamber containing control sediment and bioassay water but no amphipods.

On the day the bioassay is initiated, amphipods are distributed among all chambers so that each receives 20 individuals. It is usually not logistically possible to distribute amphipods to all chambers at the same time, so it is necessary to select a portion of the chambers (as many as 15) to be processed together. The exact number of chambers to receive amphipods at one time is dependent on the size and design of the experiment. At least one replicate from each treatment, including control and reference area sediment, is processed at a time if possible. Otherwise, selection is random.

Amphipods are removed from the holding sediment using a sieve (1.0-mm for *R. abronius* and *E. estuarius*, 0.5-mm for *A. abdita*), and then transferred to sorting trays. About one-third more fingerbowls are removed from the holding tank at one time than are required for the number of chambers. This allows selection of active, apparently healthy animals for the bioassay. Amphipods are removed from the sorting tray and sequentially distributed among clean 10-cm fingerbowls each

containing 150 mL of bioassay seawater without sediment. The number of amphipods distributed to each fingerbowl is recounted by transferring them to a separate fingerbowl.

Amphipods are added to the bioassay chambers by placing a black plastic disk on the seawater surface and gently pouring the entire contents of the fingerbowl into the beaker. The fingerbowl is washed with bioassay water to remove adhering amphipods. The seawater level is brought up to 950 mL with bioassay water, and the disk is removed and rinsed between samples. Amphipods are allowed to bury in the sediment and any that are floating on the seawater surface are pushed down with the edge of the beaker cover or a clean glass rod. For *R. abronius* and *E. estuarius*, after 15 minutes amphipods that have not buried are removed and replaced. Normally, less than 1 percent of the animals will fail to bury in 1 hour. *A. abdita* should be given one hour to burrow into the sediments.

Monitoring—If samples for chemical analysis are desired, seawater and sediment samples can be taken from chambers at the initiation of the bioassay. A small quantity of seawater can be taken from chambers at the initiation of the bioassay, but chemistry beakers have to be sacrificed to obtain sediment samples. This is accomplished by siphoning the overlying seawater without disturbing the sediment surface and then taking appropriate sediment aliquots for chemical analyses. It is not necessary to add amphipods to chemistry beakers that are sacrificed at the initiation of the bioassay, but amphipods are added to those sacrificed later. Certain sediment and water quality variables (e.g., dissolved oxygen, pH, Eh) can be monitored by inserting analytical probes into the chemistry beakers.

During the course of the bioassay, certain observations are made daily. Temperature in the chamber set up for this purpose is monitored. Lighting and aeration systems are checked. Each chamber is carefully examined but not disturbed except for the temporary removal of the aeration pipette and watchglass. Notes are made on sediment appearance and unusual conditions. The number of amphipods that have emerged from the sediment, either floating on the water surface or lying on top of the sediment, is recorded. Amphipods that have emerged are not removed, even if they are dead. These data are used to document the temporal pattern of emergence. Amphipods trapped by surface tension at the water surface are gently pushed down with a clean instrument (e.g., pipette, glass rod, beaker cover).

Termination—The bioassay is terminated after 10 days of exposure. After daily observations are recorded, the contents of the bioassay chambers are sieved through a 0.5-mm screen. Material retained on the screen is placed in clean bioassay water in a sorting tray. The numbers of live and dead amphipods are recorded. The sum of these numbers may not always equal 20 because of death and subsequent decomposition of amphipods. An amphipod is counted as alive if there is any sign of life (e.g., pleopod twitch observed under magnification, response to gentle prodding with a clean instrument).

If the reburial endpoint is to be evaluated, amphipods that survive the test are transferred to dishes containing a 2-cm layer of negative-control sediment and observed under constant illumination. The numbers of individuals able to bury after an exposure period of 1 hour is then recorded. There is no reburial endpoint in the *Ampelisca* test.

Experimental Design

Logistics—A typical sediment bioassay involves about 50–60 bioassay chambers. Collection and preparation of animals, sediment, and seawater requires at least four people for 2 days. Three or four people are required on the days experiments are initiated and terminated. One person can monitor the experiment in progress.

Controls—Five replicates of the amphipod collection-site control sediment are included in all bioassays. These comprise a negative (clean) control that allows comparisons among experiments and among laboratories of the validity of the procedures used in individual investigations. In the negative control, mean mortality should be ≤10 percent and individual replicate mortality should be ≤20 percent for the test to be considered valid. Experiments in which contaminants are added to sediment may require additional solvent control replicates to determine effects of solvent addition.

A positive (toxic) control is also required for all testing. This involves determining 96-hour LC_{50} values for *R. abronius*, *E. estuarius*, or *A. abdita* exposed to a reference toxicant in clean, filtered seawater without sediment (following standard bioassay procedures and under the same general test conditions as the sediment bioassays). Such data are necessary to determine the relative sensitivity of the animals (e.g., seasonal difference in sensitivity) for each test series to ensure comparability of the data. The commonly used reference toxicant is reagent-grade cadmium chloride. For *R. abronius*, Swartz et al. (1986) determined a 96-hour LC_{50} of 1.61 mg/L for cadmium chloride. The LC_{50}s for *R. abronius* and *A. abdita*, using data from the Army Corps of Engineers DAIS database, are 0.79±0.48 mg/l (n=42) and 0.49±0.42 mg/l (n=9) cadmium, respectively (Army Corps of Engineers personal communication, 1994). Acute lethality results for a reference toxicant must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and one above the LC_{50}. Statistical procedures for the LC_{50} estimate are given in APHA (1985) and ASTM (1989).

Reference Area Sediment—The design of field surveys typically includes a reference sediment involving five replicate laboratory tests of samples from an area believed to be free from sediment contamination. This provides a site-specific basis for comparison of potentially toxic and nontoxic conditions while controlling for the effects of exposing amphipods to non-native sediments. The grain size composition (as measured by percent silt plus clay and percent gravel) of the reference area sediment should be as similar as possible to that of the test sediment. Organic carbon content of reference area sediment should also be matched with the test sediment as closely as possible. However, it should be recognized that matching of organic carbon content may not be warranted in cases where pollution (e.g., from pulp mills, sewage outfalls, combined sewer overflows) is responsible for high organic content of test sediments.

DeWitt et al. (1988) found that sediments having a high percentage of fine-grained material could increase the mortality rate of *Rhepoxynius abronius* in the absence of apparent chemical contamination. DeWitt et al. (1988) developed a regression model to predict the relationship between amphipod mortality and sediment grain size. In their approach, test results (i.e., mean mortality values) that lie outside the 95-percent prediction limit developed from reference area data are considered indicative of chemical toxicity. A similar model can be applied to the *E. estuarius* bioassay (DeWitt et al. 1989).

Response Criteria—Endpoints examined in the amphipod bioassay include mortality, emergence from sediment, and for *R. abronius* and *E. estuarius* failure to rebury in sediment at the end of the exposure period. Data on emergence and reburial are used to monitor sublethal behavioral responses of the amphipods during (i.e., emergence) and after (i.e., reburial) the 10-day exposure. Mortality after 10 days of exposure is the primary criterion of toxicity. An estimate of total effective mortality may also be calculated by summing percent mortality and percent failure to rebury (based on the starting number of amphipods (20), not the number alive at the end of the test). Each of these response criteria must be monitored in a "blind" fashion; that is, the observer must have no knowledge of the treatment of the sediment in the chambers. This is accomplished through randomization of beaker numbers.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Water quality measurements during testing [i.e., dissolved oxygen, temperature, salinity, pH, sulfides (optional), and ammonia (optional)]
- Daily emergence for each chamber and the 10-day mean and standard deviation for each treatment
- Failure of *R. abronius* or *E. estuarius* to rebury (optional) for each chamber and the mean and standard deviation for each treatment
- 10-day mortality and total effective mortality (optional) in each chamber and the mean and standard deviation for each treatment
- Interstitial water salinity for control, reference, and test sediments
- 96-hour LC_{50} values with reference toxicants (results for metallic compounds should be reported in terms of the metal ion rather than as the weight of the whole salt)Any problems that may have influenced data quality.

BIVALVE LARVAE SEDIMENT BIOASSAY

USE AND LIMITATIONS

The bivalve larvae bioassay technique is described in *Standard Methods* (APHA 1985) and by ASTM (1989) as a rapid and reliable indicator of environmental quality. Pacific oysters (*Crassostrea gigas*) and blue mussels (*Mytilus edulis*) are recommended for testing. During the first 48 hours of embryonic development, fertilized oyster and mussel eggs normally develop into free-swimming, fully shelled larvae (prodissoconch I). Failure of the eggs to survive or the proportion of larvae developing in an abnormal manner have been used as the primary indicators of toxicity. A combined mortality and abnormality endpoint may also be calculated from the number of normal surviving larvae.

This sediment bioassay can be used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The two species recommended for testing may show different levels of sensitivity to various contaminants. Therefore, the quantitative results for corresponding endpoints may not be strictly comparable between the two species. Nevertheless, results of statistical comparisons of test sediments with reference area sediments based on the two bivalve species as well as the related echinoderm embryo bioassay may be considered interchangeable for some purposes (e.g., regulatory decision-making).

The bivalve larvae bioassay probably can be used in sediments that have interstitial salinities less than 1 ppt, as the sediments are mixed and equilibrated with seawater prior to testing. However, because further testing is required to determine the validity of using this technique with such low salinity sediments, this bioassay is not recommended for sediments that have an interstitial salinity of less than 10 ppt. In addition, the following caveats apply:

- Bivalve larvae such as those of *C. gigas* normally reside in the water column and are not intimately associated with sediments. Hence, this bioassay is primarily an indicator of the relative toxicity among different samples because its direct ecological significance with respect to *in situ* sediments has not yet been established.

- Spawning of *C. gigas* occurs naturally in the Puget Sound area in summer. The natural spawning period for *M. edulis* is late spring to early summer. Both of these bivalves can be induced to spawn at other times of the year, but may show decreased viability of gametes. Gamete viability may also vary depending on the brood stock used. Accordingly, a positive control is required. This should comprise 48-hour LC_{50} and EC_{50} measurements with a reference toxicant in seawater only.

- Relative sensitivity of the three endpoints (percent mortality, percent abnormality, and

percent combined mortality/abnormality) to toxic chemicals, natural chemical factors (e.g., total organic carbon, paralytic shellfish poison) and physical factors (e.g., suspended sediment) has not been thoroughly evaluated. However, caution should be exercised in utilizing *C. gigas* larvae in sediments known to have a high proportion of clays and silts (EPA 1993).

- High mortalities in the seawater control and/or reference sediment tests may be occasionally observed. The cause of such mortality is unknown, but may be related to natural factors that reduce embryo quality.

- It is possible that abnormalities induced during testing may be underestimated due to poor recovery of living abnormal larvae from the sediments. However, investigators have found that recovery of abnormal larvae from sediment is not worth the effort.

FIELD PROCEDURES

Collection

Both test and reference area sediment should be collected in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section titled *General QA/QC Guidelines*. Each jar should be filled completely with sediment to exclude air. A minimum sediment sample size of 18 grams for each bioassay chamber is recommended for both kinds of sediment. Because five replicate tests are conducted for each field sample, and additional sediment is used for water quality monitoring, a minimum sediment sample size of 200 grams is recommended for each station.

Processing

Both test sediment and reference area sediment should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The following procedures apply equally to larvae of both *C. gigas* and *M. edulis*, and are as described by Chapman and Morgan (1983) with the following changes incorporated:

- The salinity of test water is adjusted to 28 ppt
- Exposure time can range from 48 to 60 hours and depends on larval development in the negative controls
- Replication is increased from two to five to allow adequate statistical comparisons
- Larvae of *M. edulis* are included in the bioassay protocol
- Sediment holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.
- Seawater holding time prior to testing is set at a maximum of 2 days for field-collected and reconstituted seawater. If the seawater is not filtered or UV-treated, it should be used within 8 hours.
- Sediment resuspension in the test chambers is adequately accomplished by vigorous shaking for 10 seconds; there is no need to rotate the chambers for 3 hours at 10 rpm
- Eighteen grams of sediment is suspended in 900 mL of seawater rather than 15 grams in 750 mL
- Sediments are allowed to settle in the bioassay chamber for 4 hours prior to inoculation with embryos
- pH is not adjusted before the bioassay starts and is only monitored
- An interim oyster-specific threshold value for ammonia of 0.13 mg/L unionized ammonia has been proposed (EPA 1993); data should be qualified as possible false positives if this threshold value is exceeded
- Aeration is generally recommended and is specified for test chambers in which dissolved oxygen concentrations decline below 60 percent of saturation or when ammonia or sulfides are suspected or have been measured; all chambers should be aerated if aeration is necessary
- A positive control (reference toxicant) is required.
- Additional seawater controls are added for monitoring the stage of larval development
- For test termination, three 10-mL aliquots should be taken; one is counted and two are preserved in buffered formalin for possible later use.
- Additional details provided by ASTM (1989) for conditioning and spawning adults are included.

Bioassay Species

The species selected for testing depends on the availability of brood stock and spawning success during recent bioassays or pilot tests. For a given test or series of related tests, adult bivalves (brood stock) should be obtained from the same source: either commercial rearing facilities (oysters) or a chemically uncontaminated area (mussels). If brood stock is obtained from a commercial source, the original collection area should preferably be identified. For collection of mussels, it is recommended that the bissel threads be clipped rather than pulled loose from the native substrate, as mussels are likely to die if the bissel gland is pulled out during brood stock collection. Brood stock should be sexually mature individuals with normal, well-developed shells. Within 24 hours of collection or purchase, adults should be transported to the test laboratory and placed into flowing seawater similar in character to that from which they were taken. Rough handling, extended periods of desiccation, and abrupt changes in temperature, salinity, or other water quality variables must be avoided as these may induce premature spawning or render the stock useless for later controlled spawning or both. Upon receipt, adults should be cleaned of fouling organisms and detritus and placed in flowing seawater for conditioning.

Adult bivalves are held at recommended conditioning temperatures to stimulate final maturation of the gametes. The desired conditioning temperature (20 ± 1°C for oysters and 16 ± 1°C for mussels) and salinity (28 ± 1 ppt) should be attained gradually at increments not exceeding 2°C/day and 5 ppt/day. Conditioning may extend from a few days to several weeks depending on the physiological and gametogenic status of the adults. The length of the conditioning period is determined empirically by periodic sacrificial examination and spawning of representative individuals. Adults should be spawned or discarded within 2-3 weeks after attaining acceptable maturity because gamete quality will deteriorate rapidly with excessive conditioning. Adults should be provided with an adequate supply of natural or cultured phytoplankton. Natural seawater flow should be about 28 liters/hour per individual adult. ASTM (1989) describes procedures for maintaining holding tanks. Procedures for inducing spawning in bivalves, enhancing the quality of gametes, and preparing embryos are described in ASTM (1989).

Reference Sediment

The bivalve larvae bioassay is conducted with reference area sediment in addition to seawater controls. Reference area sediment typically consists of material collected from an area documented to be free of chemical contamination and nontoxic to bivalve larvae. The reference sediment grain size should be as similar as possible to the test sediment (see section on Puget Sound Reference Areas).

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt and temperature of 20 ± 1°C for oysters and 16 ± 1°C for mussels. Seawater should be collected from uncontaminated areas (e.g., deep or offshore waters) to avoid contamination and should be held at ≤20°C for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1989) should be held at ≤20°C for no longer than 2 days before use. The bioassay seawater must be uncontaminated and of acceptably low toxicity. The biological criterion of acceptability is that the larvae, spawned by adults in the dilution water, must attain a minimum of 70% normally developed larvae.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with 0.45-µm pore diameter. If necessary, salinity of the bioassay water is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salt, brine prepared by freezing, or reagent grade chemicals (ASTM 1989). Artificial seawater is prepared within 2 days of use and is stored in clean, covered containers at the requisite temperature.

Facilities and Equipment

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. The bioassay chamber is a 1-liter glass bottle with a screw-top lid. Bioassays are conducted at 20 ± 1°C for oysters and 16 ± 1°C for mussels, with the bottles in shallow water baths, incubators, or temperature-controlled rooms.

If adults are to be conditioned for spawning out of season, a continuous supply of temperature-controlled, aerated seawater is needed. Laboratory facilities should be well-ventilated and free of organic vapors. Holding and conditioning chambers preferably should not be in a room in which toxicity tests are conducted, stock or test solutions are prepared, or equipment is cleaned. Air used for aeration should be free of organic vapors, oil, and water. Raw seawater can be used for holding and conditioning, but feeding the adults a natural or cultivated alga is necessary to deter starvation. The flow rates used for adult conditioning must be high enough (typically >28 liters/hour/individual) to prevent water quality degradation and provide as much food as possible to the adults.

Tanks and trays are necessary for holding the adults, and a water bath, incubator, or temperature-controlled room is necessary during the bioassay. Adult holding and conditioning tanks should be cleaned several times each week to prevent accumulation of organic matter and bacteria. Dead specimens should be removed immediately and the tanks cleaned. The tanks should be cleaned with detergent and rinsed with clean seawater, and if microbial growth is present, rinsed with 200 mg/L of hypochlorite and then seawater. With enriched waters and elevated conditioning temperatures, more frequent cleaning may be required.

Bioassay Procedure

Initiation—Adult bivalves, conditioned as necessary in the laboratory, are induced to spawn with selected thermal and biological (i.e., sperm) stimulation. Selected densities of the resulting embryos are exposed to the test or reference area sediments for 48 hours, during which the embryos normally will develop into prodissoconch I larvae. A slightly longer exposure period may be used if necessary to achieve adequate development of larvae in seawater controls. Exposure time should not exceed 60 hours for an acceptable test. Data from tests with longer exposures (>48 hours) may not be comparable to those from tests conducted using the standard 48-hour exposure. Toxicity test endpoints are based on abnormal shell development and larval death.

The bivalves are spawned by rapidly raising the water temperature to 5-10°C above the conditioning temperature. Individuals are additionally stimulated to spawn by the addition of sperm from a sacrificed or naturally spawned male.

Spawning is conducted by placing the bivalves in individual, clean Pyrex™ dishes containing filtered, ultraviolet (UV)-treated seawater. Fertilization is accomplished within 1 hour of spawning by combining eggs and sperm (i.e., at a concentration of 10^5–10^7 sperm/mL) in a 1-liter Nalgene beaker. The fertilized eggs are then washed through a 0.25-mm Nitex screen to remove excess gonadal material and suspended in 2 liters of filtered, UV-treated seawater at incubating temperature. The embryos are kept suspended by frequent agitation using a perforated plunger, and used in the bioassay within 2 hours of fertilization. When microscopic examination of fertilized eggs reveals the formation of polar bodies, egg density is determined from triplicate counts of the number of eggs in 1.0-mL samples of a 1:99 dilution of homogeneous egg suspension.

Sediment bioassays are conducted in clean, 1-liter glass bottles. Five replicate tests are conducted for each field sample. An additional bioassay chamber is prepared for water quality monitoring. Eighteen grams (wet weight) of the appropriate sediment is added to each bottle and a volume of 900 ml filtered or UV-treated seawater (28 ± 1 ppt salinity) is added to all containers. The reference area sediment chambers each contain 18 grams of clean sediment. In addition, negative and positive controls for determination of LC_{50} and EC_{50} are prepared consisting of clean seawater without sediment.

The sediments are suspended by vigorous shaking for 10 seconds and the suspended sediments are allowed to settle for 4 hours prior to addition of larvae. No additional agitation is provided. The seawater controls are treated similarly except for the lack of sediments.

Within 2 hours of fertilization, each container is inoculated with 20,000–40,000 developing embryos to give a concentration of about 20–40/mL. The containers are covered and incubated for 48 hours (or longer if required) at 20 ± 1°C for oysters and 16 ± 1°C for mussels under a 14-hour light:10-hour dark photoperiod. Aeration of test chambers generally is recommended during the bioassay. Moreover, if the dissolved oxygen concentration in any test chamber declines below 60 percent of saturation or if high levels of ammonia or sulfides are suspected or measured, the water in that chamber should be aerated gently for the remainder of the test. If aeration is necessary, all chambers should be aerated and at a rate of approximately 100 bubbles per minute. A random numbering method should be used to distribute the chambers in the water bath (or incubator or cold room).

The mean embryo concentration at 0 hours should be determined by collecting five replicate 10-mL samples from control containers and preserving them in 5-percent buffered formalin. This method of determining the initial embryo concentration is one of three methods recommended by ASTM (1989) for larval bioassays of water and elutriates. Because the method recommended for the present bioassay does not rely on direct assessments of embryo densities in the test chambers, the resulting density estimates have an unquantified error component associated with them. This error reduces the reliability of larval mortality estimates and may thereby influence the results of statistical analyses. However, it does not affect larval abnormality estimates because they are based on known numbers of survivors.

Monitoring—Temperature, pH, salinity, and dissolved oxygen are measured daily in the replicates prepared specifically for monitoring water quality. Measurements are taken just prior to introduction of the embryos to the bioassay chambers at the same time each day until the conclusion of the bioassay.

Measurements of conventional water quality variables (e.g., sulfides, ammonia) should be made at the start and termination of the bioassay and additionally monitored if high levels are found (EPA 1993).

Termination—The bioassay is terminated when greater than 95 percent of the embryos in the duplicate seawater control have reached the prodissoconch I stage (approximately 48–60 hours). Once this stage has been achieved in the control chambers, final water quality measurements are recorded and the test is terminated. The bioassay is terminated in the following manner. The water and larvae overlying the settled sediment in each container are carefully poured into a clean 1-liter beaker. This water is then mixed with a perforated plunger, and 10-mL aliquots of the well-mixed sample are removed by pipette and placed in 10-mL screw-cap vials. The contents of each vial are preserved in 5-percent buffered formalin.

Preserved samples (equal in volume to those containing 200–400 larvae in controls) are examined in Sedgewick-Rafter cells. Normal and abnormal larvae are enumerated to determine percent survival and percent abnormality. A minimum sample size of 20 living larvae in each of the five replicate bioassay chambers for test sediment and reference area sediment and 100 larvae in each replicate chamber for the seawater control should be scored for abnormalities. Percent survival for each replicate bioassay chamber is based on the number of normal larvae surviving relative to the mean number of normal survivors in the seawater controls. Larvae that fail to transform to the fully shelled, straight-hinged, D-shaped prodissoconch I stage are considered abnormal. Percent abnormal for each replicate bioassay chamber is based on the number of survivors that are abnormal. Definitions of normal development specified at the PSDDA larval bioassay workshop should be followed. These definitions include the following:

- An uninterrupted shell must be formed around the margin; any indication that the shell cannot close (e.g., chips or knobs) constitutes an abnormality. Classification of open shells or shells seen in other than side view should be made on best professional judgment. Empty shells that are complete count as normal, because they developed successfully to the shelled stage, no matter what happened thereafter.

- Larvae classified as normal must have a straight hinge by termination of experiment. If larvae have not reached D or prodissoconch I stage by the end of the exposure time (set by the duplicate sacrificial control vessel) they are considered abnormal.

Controls

Five replicates of the seawater control are included in all bioassays. These comprise negative (clean) controls that allow comparison among experiments and among laboratories of the validity of the procedures used in individual investigations. At least 70 percent of the larvae must the 48-hour exposure with seawater alone. Experiments in which contaminants are added to sediment may require control replicates to determine effects of solvent addition.

A positive (toxic) control is also required. This involves determining 48-hour (or longer if required) LC_{50} and EC_{50} values for bivalve larvae exposed to reference toxicants in clean, filtered or UV-treated seawater without sediment [following standard ASTM (1989) bioassay procedures and under the same general test conditions as the sediment bioassays]. Such data are necessary to determine the relative sensitivity of the larvae. Two commonly used reference toxicants are reagent-grade cadmium chloride and sodium dodecyl sulfate. Either of these reference toxicants may be used, but the results must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} or an EC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and EC_{50} and one above the LC_{50} and EC_{50}. Statistical procedures for the LC_{50} and EC_{50} estimates are given in APHA (1985) and ASTM (1989).

Reference Area Sediment

The design of field surveys may include a reference sediment from an area known to be free from chemical contamination. This provides a basis for comparison of potentially toxic and nontoxic conditions.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Source, qualitative condition, and holding time of brood stock
- Information on how the brood stock were collected
- All water quality measurements [e.g., dissolved oxygen, temperature, salinity, pH, sulfides (optional), ammonia (optional)]
- Individual replicate and mean and standard deviation data for larval percent mortality after 48-hour exposure
- Individual replicate and mean and standard deviation data for larval percent abnormality after 48-hour exposure
- Individual replicate and mean and standard deviation data for larval percent combined mortality and abnormality after 48-hour exposure (optional)
- 48-hour LC_{50} and EC_{50} values for reference toxicants (with results for metallic compounds reported in terms of the metal ion, not as weight of the whole salt)
- Data on stocking density (number/ml), stocking aliquot size (ml), and initial count data for seawater controls

- Any problems that may have influenced data quality.

Mortality = 100 x (1 - (no. of surviving test larvae / no. of control larvae))

Abnormality = 100 x (1 - (no. of abnormal larvae / no. of normal and abnormal survivors))

Combined larval mortality/abnormality = 100 x (1 - (no. of surviving normal larvae / no. of embryos inoculated))

ECHINODERM EMBRYO SEDIMENT BIOASSAY

USE AND LIMITATIONS

The echinoderm embryo bioassay is described by Dinnel and Stober (1985) as a rapid and sensitive technique for assessing the toxicity of marine sediments. Sand dollars (*Dendraster excentricus*), purple sea urchins (*Strongylocentrotus purpuratus*), and green sea urchins (*S. droebachiensis*) are the recommended species for testing. During the first 48–96 hours of embryonic development, fertilized echinoderm eggs normally develop into the pluteus stage. Failure of the eggs to survive and the proportion of larvae developing in an abnormal manner are used as indicators of toxicity. A combined mortality and abnormality endpoint is calculated from the number of normal pluteus larvae. Unlike the bivalve larval test, a "mortality" endpoint cannot be used because dead embryos rarely disintegrate during the test as do oyster or mussel embryos.

The echinoderm embryo bioassay can be used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The three species recommended for testing may show different levels of sensitivity to various contaminants. Therefore, the quantitative results for corresponding endpoints may not be strictly comparable between the three species. Nevertheless, results of statistical comparisons of test sediments with reference area sediments based on the three echinoderm species, as well as the related bivalve larvae bioassay, may be considered interchangeable for some purposes (e.g., regulatory decision-making).

The echinoderm bioassay probably can be used in sediments that have interstitial salinities less than 1 ppt, as the sediments are mixed and equilibrated with seawater prior to testing. However, because further testing is required to determine the validity of using this technique with such low salinity sediments, this bioassay is not recommended for sediments that have an interstitial salinity of less than 10 ppt. In addition the following caveats apply:

- Echinoderm larvae normally reside in the water column and are not intimately associated with sediments. Hence, this bioassay is primarily an indicator of the relative toxicity among different samples because its direct ecological significance with respect to *in situ* sediments has not yet been established.

- Spawning of *Strongylocentrotus* spp. occurs naturally in the Puget Sound region from December to April. The natural spawning period for *D. excentricus* is from April to October. Echinoderms can be induced to spawn at other times of the year, but may show decreased viability of gametes. Gamete viability may also vary depending on the brood stock used. Accordingly, a positive control is required. This should comprise 48-hour LC_{50} and EC_{50} measurements with a reference toxicant in seawater only.

- Relative sensitivity of the two endpoints (percent abnormality and percent combined mortality/abnormality) to toxic chemicals, natural chemical factors (e.g., total organic carbon, paralytic shellfish poison) and physical factors (e.g., suspended sediment) has not been thoroughly evaluated. It has been shown that larvae of *D. excentricus* do not show an adverse response to increasing silt and clay fractions, and under conditions of expected high silts and clay, the sand dollar test is preferable to the oyster larvae test (EPA 1993).

- High mortalities in the seawater control and/or reference sediment tests may be observed occasionally. The cause of such mortality is unknown, but may be related to natural factors that reduce embryo quality.

- It is possible that abnormalities induced during testing may be underestimated due to poor recovery of living larvae from the sediments. However investigators have found that recovery of abnormal larvae from sediment is not worth the effort.

FIELD PROCEDURES

Collection

Test Animals—All recommended echinoderm species can be collected off the coast of Washington. Purple sea urchins can be found in the intertidal zone (or the shallow subtidal zone in the Strait of Juan de Fuca) and are usually ripe from December through March in Washington waters. Green sea urchins occur in the shallow subtidal zone and are usually ripe from January through April. Sea urchins should be collected with care to avoid injury from the sharp spines. Sand dollars are the preferred test species during the summer months as they are in spawning condition from about April through October. Sand dollars can be collected by hand on many Puget Sound beaches during low tide. All animals should be collected from uncontaminated areas.

Sediment—Both reference area and test sediment should be collected in glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely with sediment to exclude air. A minimum sediment sample size of 18 grams for each bioassay chamber is recommended for both kinds of sediment. Because five replicate tests are conducted for each field sample and additional sediment is used for water quality monitoring, a minimum sediment sample size of 200 grams is recommended for each station.

Processing

Sediment—Both control and test sediment should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

The following procedures are synthesized primarily from Dinnel and Stober (1985) and ASTM (1994). The following changes were incorporated:

- The salinity of test water is adjusted to 28 ppt
- Seawater temperature during the bioassay is maintained at 15 ± 1°C for all test species
- Exposure time can range from 48 to 96 hours and depends on larval development in the negative controls
- Replication is increased from three to five to allow adequate statistical comparisons
- Sediment holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.
- Seawater holding time prior to testing is set at a maximum of 2 days for field-collected and reconstituted seawater
- Sediment is included in each test chamber
- Sediment resuspension in each test chamber is adequately accomplished by vigorous shaking for 10 seconds
- Sediment is allowed to settle in test chambers for 4 hours before addition of fertilized eggs
- pH is not adjusted before the bioassay starts and is only monitored
- Aeration is specified for test chambers in which dissolved oxygen concentrations decline below 60 percent of saturation or if sulfides or ammonia are present. If aeration is required, all test chambers should be aerated at a rate of approximately 100 bubbles per minute
- A positive control (reference toxicant) is required
- Additional seawater controls are added for monitoring the stage of embryo development
- A single endpoint of combined mortality and abnormality should be calculated and reported (both should be measured)

- An ammonia testing criterion of 0.014 mg/L unionized ammonia has been proposed for the echinoderm test (EPA 1993); data may be qualified as a possible false positive response if

unionized ammonia values are greater than or equal to 0.04 mg/L

- For test termination, three 10-mL aliquots should be taken; one is counted and two are preserved in buffered formalin for possible later use.

Bioassay Species

The species selected for testing depends on the availability of brood stock and spawning success during recent bioassays or pilot tests. For a given test or series of related tests, adult echinoderms (brood stock) should be obtained from the same source: either commercial harvesters or a chemically uncontaminated area. If brood stock is obtained from a commercial source, the original collection area should be identified. Within 24 hours of collection or purchase, adults should be transported to the test laboratory and placed into flowing seawater similar in character to that from which they were taken. Because epidemic spawning can occur when echinoderms are transported in seawater, test animals can be transported in ice chests containing only kelp or other moist material, and kept cool. Rough handling, extended periods of desiccation, and abrupt changes in temperature, salinity, or other water quality variables must be avoided as these induce premature spawning or render the stock useless for later controlled spawning or both. Upon receipt, adults should be cleaned of detritus and placed in flowing seawater. Sand dollars are best held on a bed of sand in flowing or well-aerated seawater.

Reference Sediment

The echinoderm embryo bioassay is conducted with reference area sediment in addition to seawater controls. Reference area sediment typically consists of material collected from an area documented to be free of chemical contamination and nontoxic to echinoderm embryos.

Bioassay Seawater

Seawater used in the bioassay is maintained at a salinity of 28 ± 1 ppt. Water temperature should be maintained at 15 ± 1°C for all test species. Seawater should be collected from uncontaminated areas (e.g., deep or offshore waters) to avoid contamination and should be held at ≤15°C for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1994) should be held at ≤15°C for no longer than 2 days before use. The bioassay seawater must be uncontaminated and of acceptable low toxicity. The recommended biological criterion of acceptability is that the larvae, spawned by adults in the dilution water, must not incur more than 30-percent combined mortality/abnormality during 48-96 hours of exposure to the bioassay seawater.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with a 0.45-µm pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salt, brine prepared by freezing or reagent-grade chemicals (ASTM 1994).

Facilities and Equipment

All laboratory glassware is cleaned according to the procedures specified in the section entitled *General QA/QC Guidelines*. The bioassay chamber is a standard 1-liter glass jar or beaker (10-cm internal diameter) covered with an 11.4-cm-diameter watchglass. The bioassay chambers are maintained at 15 ± 1°C in a shallow water bath, incubator, or temperature-controlled room with an overhead aeration source. General recommendations of ASTM (1994) should be followed for materials used for test equipment, cleaning procedures, and good laboratory practices.

If adults are to be conditioned for spawning out of season, a continuous supply of temperature-controlled, aerated seawater is needed. Laboratory facilities should be well ventilated and free of organic vapors. Holding and conditioning chambers preferably should not be in a room in which toxicity tests are conducted, stock or test solutions are prepared, or equipment is cleaned. Air used for aeration should be free of organic vapors, oil, and water. Raw seawater can be used for holding and conditioning, but feeding the adults a natural or cultivated alga is necessary to deter starvation. The flow rates used for adult conditioning must be high enough (typically >28 liters/hour/individual) to prevent water quality degradation and provide as much food as possible to the adults.

Tanks and trays are necessary for holding adults. These are placed in a water bath, incubator, or temperature-controlled room to maintain proper temperature. Adult holding tanks should be cleaned several times each week to prevent accumulation of organic matter and bacteria. Dead specimens should be removed immediately and the tanks cleaned. The tanks should be cleaned with detergent and rinsed with clean seawater, and if microbial contamination is present, rinsed with 200 mg/L of hypochlorite and then seawater. With enriched waters and elevated conditioning temperatures, more frequent cleaning may be required.

Bioassay Procedure

Initiation—Adult echinoderms, conditioned as necessary in the laboratory, are induced to spawn with chemical stimulation. Selected densities of the resulting embryos are exposed to the test or reference area sediments for 48 to 96 hours, during which the embryos normally will develop into the four-armed pluteus stage. Data from tests with longer exposures (>48 hours) may not be comparable to those from tests conducted using the standard 48-hour exposure. The toxicity test endpoint is based on failure to develop normal pluteus larvae.

Adult sea urchins are spawned by injecting 1 mL of 0.5-molar potassium chloride (KCl) through the peristomal membrane into the coelomic cavity. Sand dollars are injected with 0.5 mL of 0.5-molar KCl through the oral opening, with the syringe held at an angle. Animals are rinsed with clean seawater and inverted over individual 150–250 mL beakers filled with seawater for about 30 minutes until spawning is completed. As many as 12 females may need to be spawned to ensure an adequate quantity of eggs. The spawning beakers should be placed in a water bath or temperature-controlled room to maintain temperature at acclimation levels.

Eggs from females discharging relatively small numbers of eggs (e.g., <100,000) are discarded. The retained eggs are examined microscopically for viability and ripeness. Ripe, viable eggs are normally round, uniform in size, free of excessive debris, and appear slightly granular. Immature eggs contain a large, clear spot (the germinal vesicle) in the cytoplasm; overripe eggs are usually less circular, have inconsistent granularity of the cytoplasm, and are often associated with increased debris. If the proportion of underripe or overripe eggs in a beaker exceeds 10 percent, the eggs are discarded. Eggs that are accepted are pooled together into a 1-liter beaker and washed three times by repeatedly decanting the water above the eggs and adding 500–1,000 mL new seawater (allow the eggs to settle to the bottom of the beaker between washes). Small subsamples of eggs are counted using a dissecting microscope to determine the number of eggs per milliliter.

The solutions of sperm from males producing thick, viscous discharges are combined to provide a stock solution. Sperm density is determined by immobilizing the sperm (i.e., by heat shock or exposure to 10-percent glacial acetic acid) and counting on a hemocytometer. Fertilization should be initiated within 1 hour of spawning by adding sperm to the beaker containing the eggs, at a sperm:egg ratio of ≤2,000:1. A perforated plastic plunger is used to gently mix the contents of the beaker. Care should be taken that excessive amounts of sperm are not used. Fertilization is monitored by examining successive 1-mL aliquots microscopically and determining the percentage of eggs with a raised fertilization membrane. When greater than 90 percent of the eggs show membrane formation (about 10-15 minutes), the developing eggs are counted and the density is adjusted to 20,000–30,000 per mL, either by diluting with seawater to decrease density or decanting excess surficial water to increase density.

Test chambers should be prepared prior to spawning of the adult echinoderms to allow enough time for sediments to settle in the chambers before inoculation with the fertilized eggs. Five replicate tests are conducted for each field sample. An additional bioassay chamber is prepared for water quality monitoring. Eighteen grams of reference or test sediment is added to each chamber. Filtered or UV-treated seawater (28 ppt salinity) is added to each chamber up to 1 liter to make a final concentration in all containers of 18 grams (wet weight) of sediment per 900 ml of seawater. Each reference area sediment chamber also contains 18 grams/900ml of clean sediment. In addition, two control series are prepared consisting of clean seawater without sediment (one series is used as a duplicate, sacrificial control to monitor embryo development).

The sediments are suspended by vigorous shaking for 10 seconds and then allowed to settle for 4 hours prior to addition of the embryos. No additional agitation is provided. The seawater control chambers are treated similarly.

Within 2 hours of fertilization, a 1-mL aliquot of the solution of embryos (about 25,000 embryos) is added to each bioassay chamber using an automatic pipette. The containers are covered with a watchglass and incubated for 48 hours (or longer if required) at 15 ± 1°C under a 14-hour light:10-hour dark photoperiod. Test chambers generally are not aerated during the bioassay. However, if the dissolved oxygen concentration in any test chamber declines below 60 percent of saturation, the water in all chambers should be aerated gently for the remainder of the test. A random numbering method should be used to distribute the chambers in the water bath (or incubator or cold room).

The mean embryo concentration at 0 hours should be determined by collecting five replicate 10-mL samples from thoroughly mixed control cultures and preserving them in 5-percent buffered formalin.

This method of determining the initial embryo concentration is one of three methods recommended by ASTM (1994) for larval bioassays of water and elutriates. The other two methods include 1) direct subsampling of each test chamber after inoculation and 2) direct subsampling of the stock solution. According to ASTM (1994), the preferred method is direct subsampling of test chambers after inoculation. This method provides the best estimate of embryo densities within each chamber and the variability of densities among chambers. However, this method cannot be easily used for the present bioassay because the sediment present in each test chamber prevents the contents of each chamber from being homogenized adequately for representative subsampling. Because the method recommended for the present bioassay does not rely on direct assessments of embryo densities in the test chambers, the resulting density estimates have an unquantified error component associated with them. This error reduces the reliability of larval mortality estimates. However, it does not affect larval abnormality estimates because they are based on known numbers of survivors.

Monitoring—Temperature, pH, salinity, and dissolved oxygen are measured daily in the replicates prepared specifically for monitoring water quality. Measurements are taken just prior to introduction of the embryos to the test chambers, then at the same time each day until the conclusion of the bioassay. Measurements of conventional water quality variables (e.g., sulfides, ammonia) should be made at the start and termination of the bioassay.

Termination—The bioassay is terminated at 48 hours or when greater than approximately 90 percent of the embryos in the duplicate seawater control have reached the four-armed pluteus stage with deeply invaginated preaural arms (whichever is later and within 48–96 hours). Once this stage has been achieved in the control chambers, final water quality measurements are recorded and the test is terminated.

The bioassay is terminated in the following manner. The water and larvae overlying the settled sediment in each chamber are carefully and gently stirred to insure the larvae are suspended in the water without disturbing the sediment; and then carefully decanting up to 98 percent of the water, leaving the sediment remaining in the test chamber. The water is then mixed thoroughly using a perforated plunger and three 10-mL aliquots of the sample are removed by pipette and placed in 10-mL screw-cap vials. The contents of each vial are preserved in 5-percent buffered formalin. One 10-mL aliquot is then counted, while the other two are archived until counts are assured to be adequate for characterizing test replicates.

Preserved samples (equal in volume to those containing 200–400 larvae in controls) are examined in Sedgewick-Rafter cells. Normal and abnormal larvae are enumerated to determine percent failing to achieve a normal pluteus larva. Percent survival for each replicate bioassay chamber is based on the number of larvae surviving in each test container relative to the initial number. Percent mortality is then calculated, including correction for mortality in the seawater control. Larvae that fail to transform into clearly defined pluteus with two well-developed arms and the second pair of arms budding are considered abnormal.

Controls

Five replicates of the seawater control are included in all bioassays. These comprise negative (clean) controls that allow comparison among experiments and among laboratories of the validity of the

procedures used in individual investigations. At least 70 percent of the larvae must achieve a normal pluteus stage. Experiments in which contaminants are added to sediment may require control replicates to determine effects of solvent addition.

A positive (toxic) control is also required. This involves determining 48 to 96 hour EC_{50} values for echinoderm larvae exposed to reference toxicants in clean, filtered or UV-treated seawater without sediment [following standard ASTM (1994) bioassay procedures and under the same general test conditions as the sediment bioassays]. Such data are necessary to determine the relative sensitivity of the larvae. Two commonly used reference toxicants are reagent-grade cadmium chloride and sodium dodecyl sulfate. Either of these reference toxicants may be used, but the results must be reported along with the sediment bioassay results. Sodium dodecyl sulfate has a short shelf life, and should be made up fresh every several days. Bioassays to establish an EC_{50} involve dilution series and a control. At least one treatment should give a partial response below the EC_{50} and one above the EC_{50}. Statistical procedures for EC_{50} estimates are given in APHA (1985) and ASTM (1994). The EC_{50} for the *D. excentricus* combined endpoint, using data in the Army Corps of Engineers' DAIS database, is 10.1 ± 6.5 mg/L cadmium (n=32) (Army Corps of Engineers personnel communication, 1994).

Reference Area Sediment

The design of field surveys may include a reference sediment from an area known to be free from chemical contamination. This provides a basis for comparison of potentially toxic and nontoxic conditions. Additionally, to further ensure the integrity of reference test samples, investigators should use experienced sampling personnel and sample from the biologically active zone, avoiding anoxic sediments. The wet-sieving method (PTI, 1991; PSDDA, 1990) should be used to match reference sediments with test sediments.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this assay:

- Source, qualitative condition, and holding time of brood stock
- Stocking density (number/ml), stocking aliquot size (ml), and initial count data for seawater control
- All water quality measurements [e.g., dissolved oxygen, temperature, salinity, pH, sulfides (optional), and ammonia (optional)]

- Individual replicate and mean and standard deviation data for combined larval mortality/abnormality at termination of bioassay (should also report separate data for mortality and abnormality for comparison to previous data endpoints)

- Individual replicate and mean and standard deviation data for larval percent combined mortality plus abnormalities at termination of bioassay

- EC_{50} values for reference toxicants (with results for metallic compounds reported in terms of the metal ion, not as weight of the whole salt)

- Any problems that may have influenced data quality.

Mortality = 100 x (1 - (no. of surviving test larvae / no. of control larvae))

Abnormality = 100 x (1 - (no. of abnormal larvae / no. of normal and abnormal survivors))

Combined larval mortality/abnormality = 100 x (1 - (no. of surviving normal larvae / no. of embryos inoculated))

GENOTOXICITY: ANAPHASE ABERRATION SEDIMENT BIOASSAY

USE AND LIMITATIONS

This sediment bioassay is used to characterize the genotoxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

This bioassay can be used with any type of sediment, regardless of the interstitial salinity or grain size characteristics. However, the following caveats apply:

- The bioassay depends on a chemical extraction procedure that is specific for neutral, nonionic organic compounds. Other classes of contaminants such as metals and highly acidic and basic organic compounds are not efficiently extracted. Thus, characterization of sediment toxicity is directed towards neutral compounds such as aromatic and chlorinated hydrocarbons.

- "Natural" genotoxicity may occur in marine sediments due to the decomposition of plant species containing genotoxic substances that evolved as a means of protecting plants from parasites and predators. Thus, positive genotoxic responses may be noted in areas generally regarded as pristine.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 200 grams is recommended for each test.

Processing

Sediment samples should be stored frozen at -20°C within 8 hours of collection. Holding time should not exceed 6 months.

LABORATORY PROCEDURES

As previously mentioned, this technique depends on a chemical extraction procedure. Extraction procedures recommended are those presently used by the National Marine Fisheries Service, National Oceanic and Atmospheric Administration in Puget Sound (MacLeod et al. 1984).

The following procedure is adapted from Kocan et al. (1982), Chapman et al. (1982a,b), Kocan and Powell (1985), and Kocan et al. (1985). Accordingly, this procedure supersedes previous published methods.

Cell Cultures

Although any cell type can be used in this test, the rainbow trout gonad cell (RTG-2) is recommended because it has been used extensively for this purpose, is readily obtainable, and is easy to cultivate. It has numerous mitotic figures when growing exponentially and the mitotic cells are large in comparison to those of other species, thereby making it easy to count the damaged anaphase cells. This cell type is sensitive to a wide range of organic chemical compounds (Kocan et al. 1982) as well as complex mixtures of chemicals that may occur in marine sediments (Kocan and Powell 1985; Kocan et al. 1985). Other cells used for this purpose are the bluegill-sunfish line, human foreskin fibroblasts, newt cells, and several plant cells (in vivo). Generally these cells can be obtained from any state or federal fish disease diagnostic laboratory, from investigators at both university and federal laboratories, or from the American Type Culture Collection (Rockville, Maryland).

Sediment Extraction

Test materials must consist of substances that are compatible with the growth of cells in culture. Generally this can be accomplished using organic extracts of environmental material such as marine sediments.

Extractions should be made using pesticide-grade reagents that have been tested for toxicity to the cells prior to their use for extraction. This initial toxicity testing can be done by evaporating a volume of the reagent equivalent to that which would be used for the actual extraction, and adding this to the cultures in varying amounts dissolved in the solvent [e.g., dimethyl sulfoxide (DMSO)] to be used for cell exposure. If the maximum anticipated amount of the solvent blank to be used in the final test does not significantly affect the cell cultures, the extraction can proceed without concern about the possible toxic effect of the solvents. Once the extracts have been made, gravimetric determinations of their absolute organic content must be made so that comparable organic concentrations from each site can be used in cell cultures exposed to extracts from different locations. This is accomplished by first weighing the tube to be used for extract storage to the nearest 0.001 gram, adding the extract in the solvent, evaporating the solvent with nitrogen, and reweighing the tubes. The difference between the original tube weight and the final tube weight is the weight of the sample extract. The sample extract is then redissolved in spectral-grade DMSO so that each sample contains the same weight/volume ratio of extract (e.g., mg/mL). In this way it is possible to expose each set of cultures to exactly the same amount of organic extract, thereby

making comparisons possible on an organic content basis. If relative toxicities from site to site are

needed, one can extrapolate back to the original organic content of each sample and compare sites based on total amounts of organic compounds present. The following detailed procedures for the preparation of sediment extracts follow those of Chapman et al. (1982a,b) and MacLeod et al. (1984).

Sediment samples are frozen and stored until just prior to extraction. Each sample is then thawed and rehomogenized by thorough stirring. An aliquot of approximately 20 grams wet weight is transferred to a clean, tared beaker, dried to constant weight (80°C), desiccated, and reweighed to determine the percent water. A second aliquot (approximately 150 grams wet weight) is transferred to a tared, 315-mL stainless steel centrifuge bottle with a PTFE-lined screw-cap, and weighed. The sample is then serially extracted with pesticide-grade solvents.

Methanol (50 mL) is added to each centrifuge bottle. The bottle is tightly capped, shaken vigorously for 2 minutes, and centrifuged at 2,000 rpm for 5 minutes. The clean solvent is decanted into a 1-liter separatory funnel. The procedure is performed twice more and the methanol extracts are combined in the separatory funnel, which is then closed and covered with aluminum foil.

Next, 100 mL of a dichloromethane/methanol (2:1 v/v) solution is added to the centrifuge bottle, the cap is closed tightly, and the bottle is shaken vigorously for 2 minutes to ensure complete mixing. The bottle is then placed in a shaker table overnight (approximately 18 hours), following which the sediment is settled by centrifuging at 2,000 rpm for 5 minutes and the solvent decanted into the separatory funnel with the methanol. A second 100-mL aliquot of the dichloromethane/methanol (2:1) solution is added, the bottle is shaken vigorously, and the bottle is then placed on the shaker table for 6 hours. The sediments are again settled by centrifuging and the solvents are decanted.

The remaining sample is shaken vigorously for 2 minutes with approximately 30 mL of dichloromethane. It is then centrifuged and the solvent is decanted into the separatory funnel. Another 100 mL of dichloromethane is added to the bottle, the cap is secured, the bottle is shaken vigorously, and the bottle is placed on the shaker table overnight. The sediments are again settled by centrifuging and the solvent is decanted into the separatory funnel. A final 30-mL rinse of dichloromethane is added, the bottle is shaken vigorously, centrifuged, and decanted. The sediment is then discarded.

Approximately 500 mL of clean, distilled water is added to the combined solvents in the separatory funnel. The funnel is carefully swirled and inverted (with frequent venting) for 2 minutes. The liquid phases are allowed to separate and the dichloromethane (lower) layer is drained into a 500-mL separatory funnel. The aqueous layer is re-extracted twice with 20 mL of dichloromethane and the remainder is discarded. The dichloromethane fractions are combined in the 500-mL funnel, transferred with rinsing, back to the 1-liter funnel, and re-extracted with another 500 mL of distilled water. The dichloromethane is drained into the 500-mL funnel and the aqueous layer is extracted once more with 20 mL of dichloromethane. The latter solvent is added to the 500-mL funnel and the aqueous layer is discarded.

The dichloromethane is drained from the 500-mL separatory funnel through approximately 20 grams of combusted and washed anhydrous sodium sulfate that is held in a 30-mL glass conical centrifuge tube with the tip cut off. The effluent from this mini-column is discharged into a 500-mL Kuderna-Danish flask with a 15-mL receiver. When empty, the 500-mL separatory funnel is rinsed with 20 mL of dichloromethane, which is drained through the sodium sulfate column into the flask. The column is washed a final time with 10 mL of dichloromethane and drained into the flask.

Boiling chips are added to the Kuderna-Danish flask and a three-ball Snyder column is placed on top. The solvent volume is reduced to about 5 mL using a hot water bath. When cooled, the sides of the flask are rinsed into the receiver with dichloromethane. The receiver is removed and the contents are quantitatively transferred to a tared conical centrifuge tube with a ground glass stopper. The sample is taken almost to dryness using the hot water bath, and stored wrapped in aluminum foil in a desiccator with the stopper open slightly until a constant weight is achieved upon reweighing the tube. This weight is the amount of extractable organic material. Samples should never be taken completely to dryness, because low molecular weight volatile compounds could be lost in the process.

After weighing, the tube is closed and wrapped fully in aluminum foil, ready for anaphase aberration testing. Extracts are treated with 1 mL of spectrophotometric-grade DMSO for 24 hours with frequent stirring on a vortex mixer. The DMSO is then removed to a glass vial and used as "stock" solution. Because all extracted material is not dissolved in the DMSO during testing, the centrifuge tubes are dried and reweighed to determine the exact amount used in testing (fraction soluble). Both stock and extract solutions are stored in the dark under nitrogen until applied to the cell cultures.

Culture Conditions

RTG-2 cells as well as most fish cell lines grow in a variety of commercially available culture media. The Leibovitz L-15 medium was found to be most consistent in terms of ease of preparation, use without special buffers or carbon dioxide incubators, and long-term storage capability. This medium can be obtained from any scientific supply house that carried cell/tissue culture materials. The medium comes as a dry powder that is added to distilled water, autoclaved or filter-sterilized, and stored in a refrigerator at 4°C until it is used. Full instructions are available with the medium when it is purchased. Generally, heat-deactivated fetal calf serum is added to any culture medium at 10-percent concentration to ensure that the proper growth factors are present. This serum can also be purchased in 100-mL to 1-liter lots already sterilized from the scientific suppliers. Before the cells are placed in the medium, pH should be adjusted to 7.1–7.3 using either sodium bicarbonate or HCl. Some laboratories use HEPES buffer (either acid or base in a commercially available product) in place of the bicarbonate or HCl. This culture system differs from that originally described by Kocan et al. (1982) only because it has been modified to simplify the laboratory procedures.

Bioassay Procedure

Cells are grown and tested at 18°C on standard, clean microscope slides or on 1×5-cm coverslips in Leighton tubes, depending on the amount of test material available. The cells are placed into the culture system 1 day prior to the actual exposure to ensure that they have had a chance to attach to the glass substrate and begin growing. On the following day (18-24 hours later), the culture medium is removed and the test material is added. This should consist of normal L-15 medium dissolved in DMSO, to which the organic extract has been added. The DMSO should be from a pretested lot to ensure that it is nontoxic to the system and should not exceed 0.5 percent (v/v) of the culture medium (e.g., 5 µL/mL). This can be reduced to 0.1-percent DMSO if toxicity is a problem or if minimal DMSO is required for conservation of extract. Exposure time should be 48 hours from the time of addition of the treated medium until fixation. Damaged cells can be observed for longer periods even after the toxic substance has been removed, but the maximum response does not increase beyond 48 hours.

An initial screening test must be conducted to determine the actual extract dilutions to be used for this bioassay. Ideally, dilutions tested for anaphase aberrations comprise both the highest concentration of extract (mg/L) that permits continued cell proliferation (i.e., is nontoxic) and a second concentration one dilution lower. This method ensures that a sufficient number of mitotic figures are present to score for chromosome damage. Based on previous experience in Puget Sound, the following six extract dilutions should be prepared: 50, 25, 15, 5, 2, and 1 µg/mL. Cells are first exposed to these concentrations for each sediment extract tested, and then the concentrations that inhibit mitosis are determined. Although identical extract dilutions may not be used to test each sediment sample, all results are normalized to organic content which has been previously determined.

To determine mitotic effects and anaphase aberrations, the slides or coverslips containing the cells are removed from the culture medium and fixed in methanol:acetic acid (3:1). The methanol is absolute and the acetic acid is glacial (undiluted). Following 15-60 minutes in the fixative (no adverse effects occur if they are left longer), the slides are air dried, then placed into 3-percent Gurr's R66 Geimsa stain for 15-30 minutes. This stain is made up in Sorensen's buffer (pH 6.8). Optimum staining is determined empirically by examining the slides with a microscope at various intervals after placing them in the stain. Problems usually occur because of too little staining time rather than excessive staining. If excessive staining does occur, the cells can be destained in Sorensen's buffer. The staining system selectively stains the condensed chromosomes undergoing mitosis and has very little effect on the cytoplasm of the cell, which allows good resolution of the small fragments of chromosome that are not associated with the main chromosome bundles.

Once the cells have been stained, they are mounted on microscope slides (if on coverslips) or are covered with coverslips (if grown on slides) to facilitate microscopic examination and scoring. Slide identification labels are covered with a piece of tape to prevent observer bias while scoring. Three replicate slides are made of each exposure concentration with two concentrations for each sediment extract. Each slide is then examined at 500X to 1,000X until a minimum of 100 anaphase cells is observed and scored. In this way, there will be three replicates per dose with 100 anaphase cells per replicate.

The numbers and percents of normal and abnormal anaphases are recorded. Cells are scored as

abnormal if they contain any of the previously described chromosomal lesions reported for this test (Nichols et al. 1977; Kocan et al. 1982; Chapman et al. 1982a; Kocan and Powell 1985; Kocan et al. 1985).

Controls

Controls consist of 1) untreated cultures used as negative controls to ensure that the culture conditions (e.g., medium, serum) are not toxic to the cells, 2) a solvent blank to ensure that the residue from the solvents used during the extraction procedure is not cytotoxic or genotoxic (generally done prior to actual testing and includes the DMSO that will be used in the final solution), and 3) a positive control consisting of cultures exposed to several concentrations of a known genotoxic agent to indicate that the test system is functioning properly and does indeed respond to genotoxic substances. One possible positive control is a 0.25-µg/mL concentration of benzo(a)pyrene. This level of exposure should result in an anaphase aberration frequency of 50-65 percent (Chapman et al. 1982a).

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Initial screening data for the determination of extract concentrations
- Individual replicate and mean and standard deviation data for numbers (and percentages) of normal and abnormal anaphases observed
- Types of anaphase aberrations observed
- Frequency of anaphase aberrations observed with the positive control
- Any problems that may have influenced data quality.

MICROTOX SEDIMENT BIOASSAY - ORGANIC EXTRACT

USE AND LIMITATIONS

The Microtox bioassay is a rapid, sensitive method of toxicity testing based on light emission by the luminescent bacterium *Photobacterium phosphoreum* in the presence and absence of aqueous toxicants. The emitted light is a product of the bacterial electron transport system and thus directly reflects the metabolic state of the cells. Accordingly, decreased luminescence following exposure to chemical contaminants provides a quantitative measure of toxicity. The assay was developed for use in freshwater habitats to assess the toxicity of waterborne pollutants (Bulich et al. 1981) and has been adapted for use in the marine environment to assess toxicity of organic sediment extracts (Schiewe et al. 1985).

This sediment bioassay is used to characterize the toxicity of marine sediments. It may be used alone as a screening tool in broad-scale sediment surveys, in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments addressing a variety of sediment and water quality manipulations.

The Microtox bioassay can be used with any type of sediment, regardless of interstitial salinity or grain size characteristics. However, the following caveats apply:

- The bioassay depends on a chemical extraction procedure that is specific for neutral, nonionic organic compounds. Other classes of contaminants such as metals and highly acidic and basic organic compounds are not efficiently extracted. Thus, characterization of sediment toxicity is directed towards neutral compounds such as aromatic and chlorinated hydrocarbons.

- Extraction by an organic solvent results in exposure of test organisms to concentrations of contaminants much higher than those expected to occur in sediment interstitial water. The use of an organic extract may not mimic pore-water composition and natural exposure routes.

- Naturally occurring toxic substances may be present in and extracted from marine sediments. Hence, relatively high toxicity occasionally may be noted in areas generally regarded as free from chemical contamination.

- Luminescence can increase, rather than decrease, in some samples. At present, these samples are considered nontoxic. However, additional research is needed to determine the meaning of an increase in luminescence.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 500 grams is recommended for each station.

Processing

Sediment samples should be stored frozen at -20°C within 8 hours of collection. Holding time should not exceed 6 months.

LABORATORY PROCEDURES

Facilities and Equipment

The bioassay is performed using a Microtox toxicity analyzer system, a temperature-regulated photometer equipped with a photomultiplier. Freeze-dried bacteria, reconstitution solution (i.e., organic-free distilled water), diluent, and other necessary materials can be purchased from commercial suppliers. The test procedure is conceptually quite straightforward. However, the methodology requires careful attention to detail and requires 1-2 weeks to become technically proficient.

Sediment Extraction

As previously mentioned, this technique depends on a chemical extraction procedure. Extraction procedures recommended are those presently used by the National Marine Fisheries Service, National Oceanic and Atmospheric Administration in Puget Sound (MacLeod et al. 1984).

- Thaw sediment, decant and discard excess water. Homogenize sediment with stainless steel spoon. Discard large pebbles, shells, seaweed, wood, crabs, etc.
- Weigh out 10 ± 0.5 grams sediment to the nearest 0.01 gram and place in a dichloromethane-rinsed centrifuge bottle.
- Set aside approximately 10 grams of the homogenized sediment for the determination of dry weight.
- Centrifuge sediment sample for 5 minutes at 1,000 × G and discard water. Centrifuge bottles should be made of glass or stainless steel.
- Add 100 mL spectral-grade dichloromethane and 50 grams sodium sulfate to sediment sample. Note that spectral-grade solvents may contain some trace contaminants that

may be toxic to the test organism. Pesticide-grade solvents are preferred.

- Manually shake bottle until contents are loose and free-flowing and then roll 16 hours (overnight) on a tumbler.
- Centrifuge sediment 5 minutes at 1,000 × G and save the dichloromethane extract.
- Add another 100 mL dichloromethane to sediment and tumble for 6 hours (during day).
- Centrifuge for 5 minutes at 1,000 × G and save the dichloromethane extract.
- Repeat extraction a third time with 100 mL dichloromethane for 16 hours (overnight) and collect extract by centrifugation.
- Combine the three 100-mL portions of the dichloromethane extract.
- Add three or four boiling chips to flask containing the dichloromethane extract and attach to Snyder column.
- Concentrate extract to 10-15 mL in a 60°C water bath and then transfer it to a concentrator tube.
- Wash down the flask 2 times with 3-4 mL dichloromethane and add washings to the tube.
- Add a boiling chip to the tube and, using a tube heater, concentrate the extract to greater than 0.9 but less than 1.0 mL.
- Adjust volume to 1.0 mL with dichloromethane (at this point 0.1 mL may be removed for gas chromatography/mass spectrometry analyses).
- Add 3 mL of hexane to the remaining 0.9 mL of extract and concentrate to 2 mL.
- Remove 100 µL of extract and add to a concentrator tube containing 3 mL nondenatured ethanol.
- Place tube in heater block and concentrate to 2 mL.
- Adjust volume to 3.0 mL with nondenatured ethanol (the standard sediment extract used in Microtox testing).
- Extraction blanks are prepared using an identical procedure but without the sediment.

Bioassay Procedure

The approach to testing organic extracts uses the basic Microtox method described in the *Microtox Operating Manual* (Beckman Instruments 1982) and by Bulich et al. (1981).

- Reconstitute lyophilized bacteria with 1-mL double-distilled, charcoal-filtered water in a

Microtox cuvette and place in 4°C holding well. Bacterial suspensions should be used within 2 hours of reconstitution.

- Prepare three or more widely spaced primary dilutions [e.g., 5.0-, 0.5-, and 0.05-percent extract (v/v)] in double-distilled, charcoal-filtered water.

- Adjust concentration of each primary dilution to 2-percent NaCl by adding 0.1 mL of 22-percent NaCl per mL of diluted extract.

- Use these diluted extracts in a range-finding assay to determine an appropriate primary dilution for the definitive assay described below. The primary dilution should cause a 65-90 percent decrease in bioluminescence in 15 minutes. Methods for the range-finding assay are the same as those for the definitive assay except usually only three concentrations are tested without replication.

- For the definitive assay, two-fold serial dilutions (i.e., 6.0, 2.5, 1.25, and 0.625 percent) of extract are prepared in 2-percent NaCl. A 2-percent NaCl blank is also prepared for testing to measure spontaneous decay of light production which occurs naturally independent of treatment.

- In each of 10 test cuvettes, a 10-µL aliquot of bacterial suspension is added to 500 µL of diluent and incubated for 15 minutes in the incubation wells. This assures temperature equilibration and stability of bioluminescence.

- After 15 minutes, initial levels of light emission are measured in each of the 10 test cuvettes.

- At 30-second intervals, 500-µL aliquots of each concentration of extract are added to two of the cuvettes (i.e., two replicates each of the four extract dilutions and the saline blank). Timing is critical because bioluminescence gradually decreases over time.

- Exactly 5minutes after addition of the sediment extract, light emission is measured at 30-second intervals and in the same sequence used for extract additions in the preceding step. Light emission is measured again at 15 minutes; additional measurements are sometimes made at 30 minutes.

- Immediately after testing each sediment extract, an ethanol-only control is assayed using the same primary dilution sequence used in the sediment extract test. The ethanol-only data are used to adjust the sediment extract data for the contribution of the solvent vehicle.

Controls

- Ethanol, sodium lauryl sulfate, or other suitable reference toxicants should be used as

positive controls to assess daily bioassay performance and to determine differences in response among lots of bacteria

- Clean sediment can be evaluated as a negative control

- Bioassay repeatability is evaluated by duplicate testing (i.e., extraction and analysis) of 10 percent of the sediment extracts.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Range-finding assay results.

- Raw light emission data for each test series.

- Determination of a significant dose-response relationship by least-squares regression of the natural log of gamma values for percent decrease in luminescence on the natural log of extract concentration based on the 15-minute data.

- 15-minute EC_{50} values and 95-percent confidence intervals for each test series and for controls. Estimates of the 15-minute EC_{50}s (i.e., the concentration of extract causing a 50-percent reduction in bioluminescence) are obtained using linear regression analyses [see *Microtox Operating Manual* (Beckman Instruments 1982)]. Briefly, the percent inhibition of light emitted at each test concentration and time point is converted to a gamma value which is defined as the ratio of light lost to light remaining. Gamma values are normalized for natural decline in light production measured over time as described by Bulich et al. (1981) and further adjusted for the contribution of the ethanol vehicle. The natural log of gamma is regressed on the natural log of extract concentration and the EC_{50} is calculated from the regression equation. A statistical procedure based on Fieller's theorem (Finney 1984) is used to calculated a 95-percent confidence interval.

- Any problems that may have influenced data quality.

MICROTOX BIOASSAY - SALINE EXTRACT

USE AND LIMITATIONS

The use of saline extracts of sediment for the Microtox bioassay has been described by Williams et al. (1986). The organic and saline extract approaches both use the basic Microtox method described in the Microtox Operating Manual (Beckman Instruments 1982) and by Bulich et al. (1981). The major difference is in the preparation of test samples. Each procedure is specific with regard to the classes of contaminants that are tested for toxicity and, in general, the results for each approach can be viewed as complementary. Additional variations of the Microtox test are being developed that are based on the direct exposure of the test organisms to interstitial water or sediments (i.e., in Microtox diluent).

The saline extract Microtox bioassay procedure removes only the water-soluble fraction of sediment-adsorbed trace metals and organic pollutants from the sediments. Thus, contaminants with extremely low water solubilities [polychlorinated biphenyls (PCBs)] will tend to be partitioned almost exclusively onto sediment particles and are unlikely to occur in high concentration in the saline extract used in toxicity testing. This is a limitation of the test if exposure is primarily through ingestion of sediment, rather than contact with pore-water. Limitations of the Microtox saline-extract bioassay include the following:

- A correction factor needs to be established for changes in bacterial luminescence caused by variation among samples in sediment pore-water salinity. Although Williams et al. (1986) showed that salinity-induced changes in luminescence were negligible for sediments taken from Commencement Bay, other estuarine sediments may have a greater range of pore-water salinities and may require a salinity correction factor.

- The use of a standardized dilution series limits the calculation of EC_{50} in some cases. The 100-percent dilution presently specified in the protocol consists of approximately 58-percent sediment extract and 42-percent Microtox diluent, thereby limiting the sensitivity of the test. A range-finding test could be conducted to determine the appropriate dilution series to calculate an EC_{50}.

- The use of a saline extract may not mimic the actual pore-water composition. Alternatively, True and Heyward (1989) conducted the Microtox test on undiluted interstitial water and demonstrated a greater sensitivity than that achieved with the saline extract protocol. The assay requires further research to demonstrate the efficiency and precision of the extraction procedure and the stability of toxicity during the allowable sediment holding time (i.e., 2 weeks) and the allowable extract holding time (i.e., 2 hours). Further research into alternative techniques (i.e., analysis of full-strength saline extract or sediment pore water) is recommended.

- Luminescence can increase, rather than decrease, in some samples. At present, these samples are considered nontoxic. however, additional research is needed to determine

the meaning of an increase in luminescence.

FIELD PROCEDURES

Collection

Sediment should be collected in solvent-rinsed glass jars that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample size of 200 grams is recommended for each station.

Processing

Sediment samples should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

With slight modification, sample processing follows procedures described by Williams et al. (1986). Glass test tubes are filled with a minimum of 60 grams of sediment, sparged with high-purity nitrogen gas, tightly sealed with PTFE-lined caps, stored in the dark at 4°C, and assayed within 14 days following collection.

Preparation of Sediment Extract

- Remove 30 grams of sediment from each test tube with a stainless-steel spatula, place in 30-mL glass containers equipped with a fitted glass cap, and add 10 mL of Microtox diluent (2.0-percent NaCl w/v in double-distilled, organic-free water).
- Mix the sediment-diluent slurry for 24 hours in the dark at 4°C by gentle agitation (100 rpm) on a rotary shaker table.
- Transfer the sediment slurry to 30-mL Corex tubes and centrifuge for 15 minutes at 9,000 rpm (9,770 × G) in a refrigerated (4°C) centrifuge.
- Draw the supernatant off by pipette, place it in a clean test tube, cool on ice, and use immediately in preparation of serial dilutions for the Microtox bioassay. Supernatant may be held at 4°C for no longer than 2 hours before use.

Bioassay Procedure

- Rehydrate a vial of freeze-dried bacteria with 1.0 mL of reconstitution solution, cover with parafilm, store in a 4°C well on the Microtox analyzer, and use within 2 hours of rehydration.

- Prepare 100-, 50-, 25-, 12.5-, and 0-percent serial dilutions of the sediment supernatant in Microtox diluent. The 0-percent dilution is a reagent blank needed to measure spontaneous decay in bacterial luminescence independent of any treatment.

- In each of 10 test cuvettes, add 10 µL of the rehydrated bacterial suspension to 350 µL of diluent and incubate for 15 minutes in one of the 15°C wells on the analyzer. This ensures temperature equilibration of the bacterial suspension and stability of luminescence.

- After 15 minutes, measure initial luminescence in each of the 10 test cuvettes.

- At 30-second intervals, add 500-µL aliquots of each supernatant dilution to two of the cuvettes (e.g., two replicates each of the four test dilutions and the saline blank). Timing is critical because toxicant-induced decrease in luminescence begins as soon as the sediment supernatant is added to the bacterial suspension.

- Exactly 15 minutes after addition of the sediment supernatants, measure luminescence at 30-second intervals and in the same sequence used for supernatant additions in the preceding step.

- Calculate percent decrease in luminescence relative to the reagent blank using the formula:

$$\text{Percent Decrease} = [(Ri_o - I_t)/(RI_o)] \times 100$$

where:

I_o = initial luminescence
I_t = luminescence at the end of 15 minutes
R = blank ratio.

The blank ratio is calculated by:

$$R = B_t/B_o$$

where:

B_o = initial luminescence of the reagent blank

B_t = luminescence of the reagent blank after 15 minutes.

Controls

- Clean reference sediments used as negative controls

- Construction of a calibration curve to determine salinity-induced changes in bacterial luminescence (Bechman Instruments 1982)

- Use of a reference toxicant (i.e., phenol, sodium arsenate) to assess day-to-day performance of the bioassay and to determine differences in toxic response among lot number of bacteria. The EC_{50} for phenol using data in the Army Corps of Engineers' DAIS database is 20.1 ± 4.7 mg/L (n=56) (Army Corps of Engineers personnel communication, 1994).

- Verification of a dose-response relationship between bacterial luminescence and sediment extract concentration.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Initial and final luminescence and percent decrease in luminescence after 15-minute exposure for each concentration of supernatant (e.g., saline sediment extract) tested.

- Determination of a significant dose-response relationship by least-squares regression of the natural log of gamma values for percent decrease in luminescence on the natural log of extract concentration based on the 15-minute data.

- Fifteen-minute EC_{50} values and 95-percent confidence limits for each test series and the reference toxicant. Estimates of the 15-minute EC_{50} values (i.e., the concentration of extract causing a 50-percent reduction in bioluminescence) are obtained using linear regression analyses [see *Microtox Operating Manual* (Beckman Instruments 1982)]. Briefly, the percent inhibition of light emitted at each test concentration and time point is converted to a gamma value which is defined as the ratio of light lost to light remaining. Gamma values are normalized for natural decline in light production measured over time as described by Bulich et al. (1981). The natural log of gamma is regressed on the natural log of extract concentration and the EC_{50} is calculated from the regression equation. A statistical procedure based on Fieller's theorem (Finney 1984) is used to calculate a 95-percent confidence interval.

- Any problems that may have influenced data quality.

JUVENILE POLYCHAETE SEDIMENT BIOASSAY

OVERVIEW

This protocol is for conducting a bioassay in which the survival and change in biomass of juvenile polychaetes *(Neanthes* sp.) are determined following a 20-day exposure to test sediments. Parameters measured to determine the effects of exposure include mortality, total biomass, and average individual biomass. Sediments can be either naturally occurring, field-collected samples, or sediments that have been experimentally modified (e.g., sediment mixed with other sediment to form a gradient of sediment types or sediment to which chemicals have been added). This bioassay is conducted as a static renewal exposure, and food (i.e., TetraMarin®) is provided to the test organisms during the exposure period to promote body tissue increases. Following the 20-day exposure period, all surviving worms are collected, dried to a constant weight, and total and average individual biomass are determined.

INTRODUCTION

Neanthes sp., a marine nereid polychaete, is widely distributed throughout the world, and has been collected in New England, Florida, California, Europe, and the central Pacific Ocean (Reish 1980). Laboratory cultures of *Neanthes* have been successfully maintained since 1964. Pesch et al. (1988) reported a difference in chromosome numbers from two populations (collected in Connecticut and California, respectively) of *Neanthes*. In addition to the differences observed in chromosome numbers, differences were also noted in the morphology indicating that these populations represent different species. Although specimens from both populations have been used in testing, almost all the testing data are associated with experiments conducted with specimens from the California population. The procedures discussed in this protocol are for *Neanthes* originating from the California population.

Since 1966, various life stages of *Neanthes* have been used as bioassay organisms for a wide variety of investigations including evaluating the effects of dissolved oxygen concentrations, nutrients, salinity, temperature, metals, pesticides, hydrocarbons, and contaminated sediments on survival, growth, and reproduction. In addition, *Neanthes* has also been used to investigate the effects of mutagens (Pesch et al. 1981; Pesch and Pesch 1980) and irradiation (Jones et al. 1983) on marine organisms; an interlaboratory comparison has been conducted with a *Neanthes* 28-day flow-through seawater toxicity test (Pesch and Hoffman 1983); and a 96-hour acute sediment bioassay using *Neanthes* is currently being used for dredged material testing by the U.S. Army Corps of Engineers, Los Angeles District (Reish and Lemay 1988).

Species Sensitivity

Neanthes has been used to evaluate the toxicity of a wide variety of contaminants including metals, hydrocarbons, and multicontaminated media (i.e., sediments). Examples of the types of toxicity tests conducted with *Neanthes* and species sensitivity are presented in Table 4.

Reish (1984) summarized data on the sensitivity of *Neanthes* to metals. In comparison to other polychaetes, *Neanthes* appears to be moderately sensitive to most metals tested. Studies indicate that mercury and copper are the most toxic to *Neanthes*, followed by aluminum, cadmium, chromium, zinc, lead, and nickel.

Ecological Importance

Neanthes is distributed on the west coast from Mexico to southern California (Reish 1980). *Neanthes* has not been collected from Puget Sound. The family Nereidae is widely distributed and is a dominant taxa in intertidal and subtidal habitats. In Puget Sound, the nereid *Platynereis bicanaliculata* is a dominant member of the polychaete fauna at many sites (Lie 1968; PTI and Tetra Tech 1988a,b). *P. bicanaliculata* is morphologically similar to *Neanthes* in jaw structure and is also recorded to be an omnivore, feeding on algae and other detritus (Fauchald and Jumars 1979). Both species also build similar tubes of organic material and display similar aggressive behavior patterns (Gray 1974).

USE AND LIMITATIONS

The *Neanthes* sublethal bioassay is used to characterize the toxicity of marine sediments primarily based on worm growth. Data reported by Johns and Ginn (1990b) indicate that the level of contamination affecting juvenile growth in *Neanthes* is similar to the level of contamination that affects reproductive success. The bioassay may be used alone (e.g., as a screening tool in broad-scale sediment surveys), in combination with sediment chemistry and *in situ* biological indices, and in laboratory experiments to address various sediment and water quality manipulations. The following constraints apply:

- The bioassay should be conducted with laboratory-cultured juvenile *Neanthes*
- Modification of the protocol may be required for tests conducted at salinities (both interstitial and overlying water) less than 20 ppt.

TABLE 4. SENSITIVITY OF *NEANTHES* TO VARIOUS CONTAMINANTS

Contaminant	Lowest Concentration for Observed Effect (mg/L)	Endpoint	Test Duration	Reference
Aluminum	>2.0	Mortality	4 days	Petrich and Reish (1979)
Cadmium (as $CdCl_2$)	3	Mortality	28 days	Reish (1980)
	1	Reproduction	Life cycle	Reish and Gerlinger (1984)
Chromium (as CrO_3)	0.6	Mortality	28 days	Reish (1980)
Hexavalent chromium (as $K_2Cr_2O_7$)	0.0125	Reproduction	Life cycle	Oshida (1976)
Copper	0.1	Mortality	29 days	Pesch and Morgan (1978)
Lead [as $Pb(CH_3CO)_2$]	3.2	Mortality	28 days	Reish (1980)
	0.97	Reproduction	Life cycle	Reish and Gerlinger (1984)
Mercury (as $HgCl_2$)	0.17	Mortality	28 days	Reish (1980)
Nickel	49.0	Mortality	4 days	Petrich and Reish (1979)
Silver (as $AgNO_3$)	0.165	Mortality	28 days	Pesch and Hoffman (1983)
Zinc (as $ZnSO_4$)	1.4	Mortality	28 days	Reish (1980)
	0.32	Reproduction	Life cycle	Reish and Gerlinger (1984)
DDT	0.1	Mortality	28 days	Reish (1985)
No. 2 fuel oil	2.7	Mortality	4 days	Rossi and Anderson (1976)
South Louisiana crude oil	12.5	Mortality	4 days	Rossi and Anderson (1976)

FIELD PROCEDURES

Collection

Test Animals—*Neanthes* are not indigenous to Puget Sound and test organisms must be obtained from laboratory cultures. (See *Laboratory Procedures* section for a discussion on culturing and obtaining test organisms.)

Sediment—Control, reference and test sediments should be collected in solvent-cleaned glass containers that have PTFE-lined lids and have been cleaned according to the procedures described in the section entitled *General QA/QC Guidelines*. Each jar should be filled completely to exclude air. A minimum sediment sample collection size of 0.25 liters for each bioassay chamber is recommended for all sediment types. Because five replicate tests are conducted for each field sample, a minimum sample size of 1.25 liters is recommended for each station.

Processing

Test Animals—Not applicable.

Sediment—All sediments should be stored at 4°C in the dark. Holding time should be set according to the procedures described in the section entitled *General QA/QC Guidelines*.

LABORATORY PROCEDURES

Test Animals

Culturing—Almost all *Neanthes* used for laboratory tests come from laboratory cultures. Culturing techniques have been described by Reish (1980) and Pesch and Schauer (1988). Under laboratory conditions, the *Neanthes* life cycle is completed in 3–4 months at 20–22°C.

Cultures of adult *Neanthes* are maintained in glass aquaria under static (with monthly renewal) or flow-through water conditions. After sexually mature males and females pair, the pairs can be isolated in jars and maintained until juveniles are ready to be removed and used for testing. Eggs are laid within the worm tube, and the female dies within 2–3 days. The zygotes are cared for by the surviving male. Larvae emerge from the worm tube in approximately 3 weeks (at 20–22°C) following fertilization. Hatched larvae feed on yolk reserves until emergence from the adult worm tube. Following emergence, the juvenile worms are capable of feeding and building independent tubes. Until testing, the juvenile worms are maintained without sediment and are provided TetraMarin® (a food source) and powdered alga (either *Enteromorpha* or *Ulva* sp.). Enough powdered alga (sieved to less than 0.3 mm) should

be provided to cover the bottom of the aquarium. The powdered alga provides material for tube construction and increases survival (Pesch and Schauer 1988).

Shipping and Holding—Juvenile *Neanthes* are obtained from laboratory cultures. If test organisms are obtained from an outside source, enough time should be allotted to allow the worms to acclimate prior to starting a test. *Neanthes* can be shipped by overnight courier without significant mortality. Worms are typically packed in plastic bags containing seawater with 50 organisms per bag. Each bag should contain several fronds of dried *Enteromorpha*. This alga can be collected, dried, and stored for extended periods. Prior to use, the alga should be soaked in seawater. The bags are shipped in a hard-sided container (e.g., cardboard box). When the shipment arrives at the laboratory, the worms, still in the plastic bags, are placed in a holding aquarium containing seawater at the proper test temperature. The worms are released from the bags after temperature equilibration. The worms are maintained in the holding aquarium for 1-2 days prior to initiation of the bioassays. The holding time will provide for acclimation between the culture temperature and the anticipated testing temperature and for observation of the condition of the test organisms to ensure that the bioassay is conducted with healthy individuals.

Neanthes juveniles should be held in all-glass aquaria containing clean seawater and provided with gentle aeration [see Pesch and Schauer (1988) if flowing seawater is available]. Water temperature is maintained at 20 ± 1°C, and salinity is maintained at the salinity at which the bioassay will be conducted. Enough powdered green alga (*Enteromorpha* or *Ulva* sp.) should be provided to cover the bottom of the holding tank.

During the holding period, organisms are provided with TetraMarin® on an every-other-day basis. The amount of food provided should be calculated at approximately 8 mg (dry weight) per juvenile, but the tank should be observed following feeding to determine if the food is being consumed. If it is not being consumed, then the amount of food provided should be reduced in order to avoid potential water fouling problems. If the entire amount of food provided is being eaten, then an increase in the food ration might be appropriate.

No water changes in the holding tank are required if the worms are being maintained in the aquaria for less than 1 week. If the worms are to be maintained for a longer period, then the water should be replaced with fresh seawater once every 2 weeks. Rising salinity, due to evaporative losses during the holding period, can be compensated for by adding sufficient distilled water to lower the salinity to the desired level.

Test Animal Size—The size of juvenile worms used in the bioassays is potentially a critical factor to the eventual success of the bioassay. Worms should be 0.5–1.0 mg (dry weight) (i.e., 2-3 weeks post-emergence) to ensure that they are in a rapid growth phase during the exposure period. Under PSDDA testing, a minimum initial size of 0.5 mg is considered a warning flag if not achieved. Worms of the appropriate size are large enough to be easily handled to avoid errors in placing the correct number of worms in each exposure chamber. For consistency in aging test organisms, initiation of emergence should be considered as the point when feeding juveniles emerge from the egg case. Commencement of feeding can be identified by the presence of food particles in the digestive tract.

Feeding Requirements—Several different types of food have been used in culturing *Neanthes*, including alfalfa flour, powdered alga (*Enteromorpha* or *Ulva* sp.), TetraMarin®, and prawn flakes. Of these foods, prawn flakes and TetraMarin® appear to provide the best and most consistent growth throughout the life cycle. Because of potential problems in obtaining a consistent supply of prawn flakes, TetraMarin® should be used. TetraMarin® should be provided to juveniles maintained in holding tanks prior to testing and during the exposure period. In both cases, the worms should be fed on an every-other-day basis. The amount of food provided should be calculated at approximately 8 mg (dry weight) per juvenile *Neanthes*.

Control and Reference Sediments

Five replicates of the polychaete collection-site control sediment are included in all bioassays. These compromise a negative (clean) control that allows comparisons among experiments and among laboratories of the validity of the procedures used in individual investigations. For the *Neanthes* bioassay, sand should be used as the control sediment.

Sand was initially chosen as an appropriate control sediment based on the work of Pesch and Hoffman (1982), who used sand as a substrate in a series of experiments with *Neanthes*. They reported no significant mortality associated with maintaining the worms in sand. For the sublethal bioassay test demonstration study and subsequent testing (Johns and Ginn 1990a,b), sand collected from West Beach on Whidbey Island, Washington, was used as the control sediment. *Neanthes* maintained in West Beach sand exhibited low mortality and high percentage increases in biomass during the exposure period, indicating that West Beach sand is a suitable material for a control sediment. In addition, West Beach sand was selected because it was used as a control sediment for a number of the regulatory bioassays conducted in Puget Sound and is known to be relatively free of contaminants. However, West Beach sand was adequate only when food was supplied.

Because control sediments may differ greatly from the test sediments with respect to physical and chemical sediment characteristics (e.g., grain size and organic content), a reference sediment is also included in the bioassay series. Data from the reference sediment can be used to partition contaminant effects associated with a test sediment from those relating to the physical and chemical characteristics of the test sediment. Johns and Ginn (1990a) evaluated the influence of sediment grain size on *Neanthes* survival and growth following exposure to sediments having differing granulometry (expressed as a percentage of the silt/clay fraction in the sediment). The results of this experiment indicate that *Neanthes* are able to survive and grow in a wide range of sediment types. Johns and Ginn (1990a) also noted that statistical differences in growth could occasionally be detected in *Neanthes* exposed to widely differing sediment types, and cautioned that reference sediment used in *Neanthes* bioassays should have a similar grain size and organic content as the test sediments to avoid potential differences in organism response related to the physical characteristics of the sediment.

Nontreatment factors such as ammonia and sulfides can affect the results of sublethal bioassays such as the *Neanthes* biomass test. When such nontreatment effects occur, water quality monitoring measurements are necessary for determining the factors contributing to the expressed effect. For the *Neanthes* biomass test it is recommended that ammonia and total sulfides be measured at the beginning and end of the test. Further ammonia and sulfides monitoring is recommended prior to the first and second water renewals when initial monitoring reveals greater than 0.7 mg/L unionized ammonia or

greater than 5 mg/L total sulfides (Dillon et al. 1993).

Test Sediments

The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. Johns and Ginn (1990a) determined the 96-hour LC_{50} for *Neanthes* exposed to seawater of different salinity to be 15 ppt. Caution should be used when performing and interpreting the results of *Neanthes* bioassays conducted with sediments with an interstitial salinity of less than 20 ppt. Modification to the test sediment (e.g., mixing the sediment with high salinity water to raise interstitial salinity) or test protocol (e.g., use of high salinity seawater in the exposure chamber) might be considered when testing sediment collected from low salinity areas.

Bioassay Seawater

Seawater used in the bioassay should be maintained at a salinity of 28 ± 2 ppt and at a temperature of 20 ± 1°C. If a series of experiments is planned, then the test temperature and salinity should be the same throughout the series. The bioassay seawater must be uncontaminated.

Facilities and Equipment

Bioassay chambers are 1-liter glass containers with an internal diameter of approximately 10 cm. The chambers are covered with lids to reduce contamination of the contents and evaporation of the seawater or loss of volatiles. The bioassay chambers are maintained at 20 ± 1°C in either a shallow waterbath or in a constant-temperature room. Exposure chambers are gently aerated with air that is free of fumes, oil, and water. This air is delivered to the exposure chamber by nontoxic tubing with a glass Pasteur pipette suspended 3–4 mm below the water surface. The aeration rate should be between 150 and 300 mL/minute, or approximately 100 bubbles per minute.

Prior to use, all glassware is thoroughly cleaned, following the General QA/QC guidelines for equipment cleaning procedures.

Bioassay Procedure

Overview—The bioassays are conducted using a static renewal exposure system. Five replicate tests are conducted for each field sample. Each exposure chamber consists of a 1-liter jar or beaker containing 175 mL of sediment (which should be approximately 2 cm in depth) and sufficient seawater to bring the level up to 950 ml (Figure 1). Prior to testing, all exposure chambers are cleaned and rinsed in turn with distilled water, 10-percent nitric acid ($HN0_3$), and distilled water.

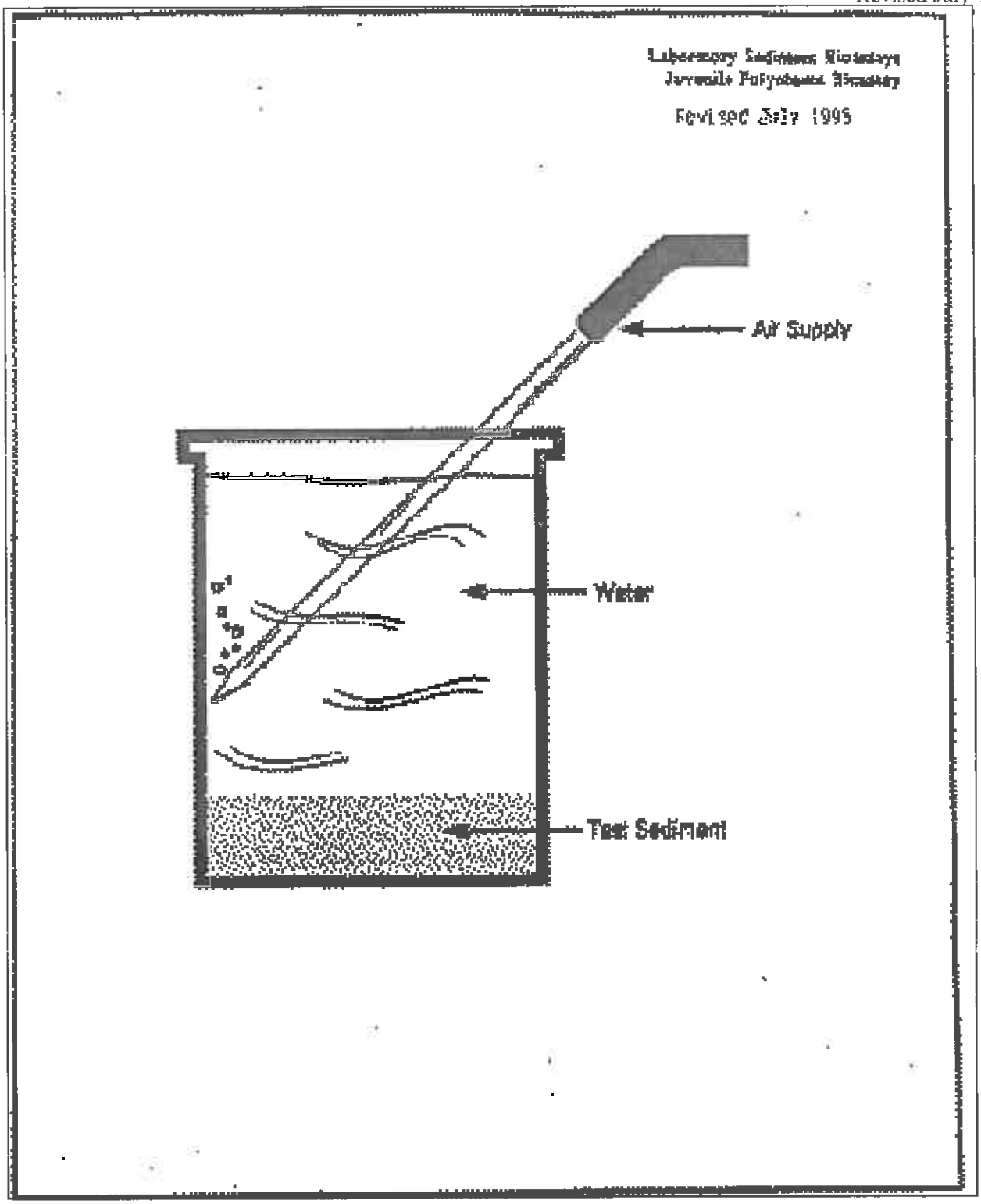

FIGURE 1. Static exposure system used for the juvenile polychaete bioassay

At the beginning of each test, five juvenile worms are randomly placed into each exposure chamber. During setup, three subsamples of worms (five worms per subsample) are randomly selected to provide an estimate of initial worm biomass.

During the exposure period, each exposure chamber is provided with 40 mg of food (i.e., 8 mg per individual) on an every-other-day basis. Every third day, one-third of the seawater in each exposure chamber is exchanged with fresh seawater. Water quality measurements taken during the exposure period include dissolved oxygen concentration, salinity, and pH. These measurements are made for each exposure chamber just prior to the seawater exchange.

Following the exposure period, the contents of each replicate chamber are sieved through a 0.5-mm screen and the number of living worms is recorded. Surviving worms are then placed in a vial containing clean seawater. After all chambers have been sieved, the surviving worms in each vial are quickly rinsed with deionized water, placed on a preweighed aluminum pan, and dried at 50°C to a constant weight. Total weights are then determined to the nearest 0.1 mg.

Initiation—Prior to initiation of a bioassay, all exposure chambers are cleaned as described above, and test organisms are acclimated. On the day before test initiation, test sediments are placed in each of the five replicate exposure chambers. Each chamber should be filled with 175 mL of sediment so that a 2-cm sediment layer is formed in the bottom of the chamber. Sediment placed in the chamber is smoothed by tapping the jar against the palm of the hand. Once the sediment is smoothed, the chamber is filled with seawater to the 950 mL level by gently pouring the water down the side of the chamber. Filled chambers are placed in a 20 ± 1°C waterbath and capped. An air line is inserted through a hole in the cap. The exposure chambers are allowed to equilibrate overnight to bioassay conditions. The photoperiod during testing should be continuous, using ambient light of low to moderate intensity. Although the intensity does not have to be measured, light levels should be similar to that obtained from fluorescent or incandescent light sources that are not placed directly over the water bath.

On the day of test initiation, juvenile worms are collected from the holding tank for distribution to the exposure chambers. The worms should be handled as little as possible. Handling should be conducted quickly and carefully so that the worms are not unnecessarily stressed. Any worms that are accidentally dropped onto hard surfaces or are injured during handling should be discarded. To prevent possible damage to the worms during handling, various handling procedures can be employed. One procedure is to use a small, fine-point paint brush to remove the organisms from the holding tank. Because *Neanthes* produce mucus over the body surface, individual worms are easily captured and transferred with this procedure. Another handling procedure is to use a wide-bore pipette with an attached bulb. Individual organisms can be collected in the pipette through suction and can be removed from the pipette using a gentle flushing action.

Individual worms, in excess of the number needed to conduct the bioassay, are transferred from the holding tank to a shallow dish containing seawater maintained at the test temperature and salinity. Worms placed in the shallow dish should be as similar in size as possible, given the size range of worms available from the holding tank. The mean size of worms used should be no smaller than 0.5 mg. Worms transferred to the shallow dish should be observed to determine that they represent the best worms available for testing (e.g., all appear healthy and represent the smallest range in size of test organisms).

Individual worms are removed from the dish and randomly placed in a plastic cup (five worms per cup) containing seawater. Enough cups are used to equal three more than the number of exposure chambers that will be used during the bioassay. Once this procedure has been completed, worms within a cup are randomly transferred to an exposure chamber by pouring the contents into the chamber. A squirt bottle containing seawater maintained at the test temperature and salinity can be used to free any worms adhering to the cup. During the transfer process, three of the cups containing worms are randomly selected and set aside. Worms from these cups are used to estimate initial total biomass. To determine initial total biomass, worms from these three cups are quickly rinsed with an isotonic, 0.9-percent (w/v) ammonium formate solution of distilled water, placed on a preweighed aluminum pan, dried at 50°C to a constant weight, and weighed to the nearest 0.1 mg.

Once worms have been placed in all of the exposure chambers, each chamber is checked to ensure that air is flowing to the chamber and that the worms have begun to burrow into the sediment. Following setup, food (e.g., TetraMarin®) is provided to each chamber. To ensure adequate distribution of the food within the exposure chamber, a small volume of seawater (i.e., 5 mL) at test temperature and salinity is added to the cup containing the preweighed food ration. Once wetted, the food is poured into the exposure chamber. Water from a squirt bottle is used to rinse the cup of any remaining food.

Following placement of the worms in the exposure chamber, initial (i.e., 1 hour) observations of burrowing should be made. If a worm, or group of worms, do not appear to be burrowing and the observer believes that the nonburrowing behavior results from factors other than sediment toxicity (e.g., reduced viability or damage to test organisms), then those organisms should be replaced.

Monitoring—During the 20-day exposure period, the test chambers are observed on a daily basis to ensure that adequate aeration is provided and to note the general status of each chamber (e.g., presence of accumulated food, burrowing activity of worms, and presence of fouling on sediment surface). On an every-other-day basis, worms in each exposure chamber are provided with food. As discussed earlier, 40 mg of food are provided to each exposure chamber. This food ration is maintained throughout the exposure period, even though mortality may occur during the test.

Every third day, one-third of the seawater in each exposure chamber is replaced. Water replacement is achieved by removing the aeration line, then siphoning one-third of the volume and carefully replacing it with fresh seawater that has been maintained at 20 ± 1°C and at the appropriate test salinity. Steps should be taken during seawater replacement to ensure that test sediments are not disturbed. One method of replacement is to add the fresh seawater by allowing the water to slowly flow down the inside wall of the exposure chamber. When the chamber is filled, the aeration line is placed back in the chamber and the air flow is adjusted to the specified level (i.e., 150 to 300 mL/minute or approximately 100 bubbles per minute).

Prior to seawater replacement, dissolved oxygen, salinity, and pH are determined in one randomly selected exposure chamber for each sample. Dissolved oxygen is determined using a dissolved oxygen electrode. Following determination of dissolved oxygen in each chamber, the electrode is thoroughly rinsed with 20 ± 1°C seawater. Salinity is determined on a small sample of seawater using a hand-held refractometer. The seawater sample for the salinity measurement is obtained with a Pasteur pipette. The pipette should be thoroughly rinsed with seawater between samples. The pH is determined with a portable pH meter and probe. As with the dissolved oxygen electrode, the pH probe is rinsed between

readings.

Termination—Following the exposure period, worms from each exposure chamber are removed from the test sediment. Two methods can be used to collect worms from each exposure chamber. In the first, surviving worms are collected by sieving the sediment through a 0.5-mm screen. The sieve should be gently shaken in a water bath rather than sprayed with water to remove the sediment. In the second method, the sediment is placed in a white enamel pan containing seawater and searched for surviving worms. Worms collected from sediment often remain in their tubes. A worm can be removed from the tube by gently prodding either end of the tube to force the worm to leave. Once out, the worms are removed using either the tip of a small paint brush or a wide-bore pipette. Following collection, the number of worms surviving is noted on data sheets.

To determine total biomass, surviving worms are quickly rinsed in isotonic 0.9-percent (w/v) ammonium formate or distilled water, placed on a preweighed drying pan, and dried at 50°C until a constant weight is attained. Total biomass is determined to the nearest 0.1 mg as the difference in weight of the aluminum pan with and without the worms. Prior to rinsing the worms, observations should be made to determine if food or sediment is present in the digestive tract. Such information may be useful in explaining changes in individual biomass occurring during the exposure period.

During the sublethal test demonstration study, a constant dry weight was attained within 24 hours. To determine when a constant weight has been achieved, several aluminum pans containing worm samples are removed from the drying oven, placed in a desiccator, and allowed to reach room temperature. Following cooling, each aluminum pan is placed on the balance and the weight is determined. Following dry weight determinations, all samples are placed back in the drying oven. After additional drying (i.e., at least 1 hour), the same samples are again removed from the drying oven, allowed to cool in the desiccator, and reweighed. When the dry weights for the samples are the same for consecutive readings (i.e., within 0.1 mg of each other), a constant weight has been attained.

Experimental Design

Logistics—A typical *Neanthes* bioassay for testing 10 sediment samples involves about 50 to 60 exposure chambers. Collection and preparation of test organisms, sediment, and seawater requires at least four people for 2 days. Three or four people are required on the days tests are initiated and terminated. One or two people can monitor a test in progress.

Controls—A control sediment and a reference sediment should be included as part of every test. The control sediment provides a nontoxic sediment to evaluate the condition of the test organisms being used in the bioassay. The reference sediment provides a test reference to partition contaminant effects associated with the treatment sediment from those relating to noncontaminant characteristics (e.g., grain size and total organic carbon).

A positive (toxic) control is also required for all testing. This involves determining 96-hour LC_{50} values for *Neanthes* juveniles exposed in clean, filtered seawater without sediment to reference toxicants (following standard bioassay procedures and under the same general test conditions as the sediment bioassays). Such data are necessary to determine the relative sensitivity of the animals (e.g., seasonal difference in sensitivity) for each test series to ensure comparability of the data. The commonly used reference toxicant is reagent-grade cadmium chloride. Reported 96-hour LC_{50} values for *Neanthes* exposed to cadmium range between 5.0 and 22.0 mg/L (Reish 1984; Johns and Ginn 1990a; Dillon et al. 1993). The LC_{50} calculated using data in the Army Corps of Engineers' DAIS database is 12.5±5.4 mg/L cadmium (n=30) (Army Corps of Engineers personal communication, 1994).

The positive control should be conducted with 10 juveniles per exposure chamber. The worms should not be fed during the 96-hour LC_{50} exposure.

The acute lethality results must be reported along with the sediment bioassay results. Bioassays to establish an LC_{50} involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC_{50} and one above the LC_{50}. Statistical procedures for the LC_{50} estimate are given in APHA (1985).

Response Criteria—Survival, total biomass (dry weight), average individual biomass (i.e., total biomass divided by the number of surviving worms), and average individual growth rate are the four response criteria that can be determined for the *Neanthes* bioassay.

Of the four endpoints, data collected to date indicate that the survival endpoint is the least sensitive to changes in level of contamination. Although survival rates of worms in each replicate have generally been similar, it should be noted that variability in percent survival within replicates could be high since each worm in a replicate represents 20 percent of the replicate survival. The total biomass endpoint is an estimate of the biomass produced by the group of worms in the exposure container. Total biomass represents an integrated measurement of lethal and sublethal effects. Thus, a reduction in total biomass could indicate that one or more worms had died during the exposure or that the growth of all worms had been reduced. Average individual biomass is an estimate of the biomass of each surviving worm. Unlike the survival and total biomass endpoints, worm survival is not integrated into the determination of individual biomass. Dillon et al. (1993) recommend normalizing to initial weight to estimate average individual growth rates. Worm survival is an important ancillary measurement and should always be considered in the interpretation of either biomass endpoint. Each of these response criteria should be monitored in a "blind" fashion; that is, the observer must have no knowledge of the treatment of the sediment in the chambers.

DATA REPORTING REQUIREMENTS

The following data should be reported by all laboratories performing this bioassay:

- Water quality measurements during testing [i.e., dissolved oxygen, temperature, salinity, pH, sulfides (optional), ammonia (optional)]
- 20-day survival in each exposure chamber and the mean and standard deviation for each treatment

- Initial total biomass (dry weight) for three groups of five worms
- 20-day total biomass (dry weight) in each exposure chamber and the mean and standard deviation for each treatment
- 20-day average individual biomass (dry weight) in each exposure chamber and the mean and standard deviation for each treatment
- Average individual growth rate (dry weight/day) in each exposure chamber and the mean and standard deviation for each treatment
- Interstitial salinity values of control, reference, and test sediments (both initial and final)
- 96-hour LC_{50} values with reference toxicant (results for metallic compounds should be reported in terms of the metal ion rather than as the weight of the whole salt)
- Any problems that may have influenced data quality.

The growth endpoint is calculated according to the following expression:

$$G = (DW_t - DW_i)/T$$

where

G = estimated individual growth rate (milligrams dry weight/day)
DW_t = estimated individual dry weight at termination (milligrams)
DW_i = mean estimated individual dry weight at initiation (milligrams)
T = exposure time (days)

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APPENDIX C

Water Chemistry Methods

Total Oil and Grease, Petroleum Hydrocarbons (heavy), Mineral Oil and Grease and Animal & Vegetable Oil and Grease in Water by Gravimetry

CAM SOP-00326

Reference Method:

The primary reference is EPA821/R-98-002 Method 1664, Revision B: N-Hexane Extractable Material (HEM; Oil and Grease) and Silica Gel Treated N-Hexane Extractable Material (SGT-HEM;Non-polar Material by Extraction and Gravimetry. Feb. 2010, with modifications)

1. Scope

This method to be used for determining total oil & grease, total mineral animal oil and grease, Animal & Vegetable Oil and Grease and Petroleum Hydrocarbons (heavy) in water (including drinking water) and wastewater samples (including most industrial wastewater and effluents) submitted to the Organic Department.

2. Principle of the Method

The total oil and grease method is for the measurement of Hexane or DCM extractable petroleum hydrocarbons from surface and saline waters, industrial and domestic wastes. It is applicable to the determination of hydrocarbons, vegetable oils, animal fats, soaps, greases and related matter.

The method is not applicable to measurement of light petroleum fuels that volatilize at temperatures below 85°C. Petroleum fuels from gasoline through #2 fuel oils are completely or partially lost in the solvent removal operation.

Some crude oils and heavy fuel oils contain a significant percentage of residue-type materials that are not soluble in Hexanes. Accordingly, recoveries of these materials will be low.

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A 1L sample is acidified to pH≤2 and serially extracted twice with Hexane or DCM. The extract is dried over sodium sulfate. The solvent is evaporated from the extract and the residue is weighed to constant weight, to give Total Oil and Grease. If the extract is to be used for determination of mineral oil and grease, the extract is re-dissolved in hexanes.

For Mineral Oil & Grease, an amount of silica gel proportionate to the amount of extractable material is added to the solution containing the re-dissolved extract to remove absorbable materials. The solution is filtered to remove the silica gel, the solvent is evaporated, and the residue is weighed for the Mineral Oil & Grease.

AKLYATED PAH

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Scientific Principle

The separation of Polycyclic Aromatic Hydrocarbon (PAH) analytes in samples is performed using gas chromatography/mass spectrometry (GC/MS). The separation is based on compound size, boiling point and polarity. Target unsubstituted PAH are quantitated against the average response factor of multipoint calibration curves of these PAH using the internal standard method. The concentration of alkylated PAH homologous series are calculated in a similar manner against standards representative of each homologous series. This is possible because each compound within a homologous series (e.g. C3 fluorenes) elutes within a relatively narrow time frame and fragments yielding a common ion (208 in the case of C3 fluorenes).

Sample extraction procedures for GC/MS analysis are as follows:

Soil**:** PAH and alkylated PAH compounds are extracted from soil samples using a solvent extraction procedure. 10-12 g of sample, as received, is mixed with 1:1 hexane:acetone in a mechanical shaker or equivalent apparatus. The efficiency of extraction is monitored by the addition of labeled PAH surrogates to the sample prior to extraction. After the mechanical extraction, acetone is displaced from the extract with organic free deionized water. For alkylated PAH determination, the aliphatics which could interfere are removed by a silica gel cleanup.

Water: PAH compounds are extracted from water samples using a liquid-liquid extraction with hexane. A known volume of sample is brought to a pH of >11 and, after the addition of the hexane solvent, is placed in a rotary tumbler or equivalent apparatus. The efficiency of extraction is monitored by the addition of labelled PAH surrogates to the sample prior to extraction. For alkylated PAH determination, the aliphatics which could interfere are removed by a silica gel cleanup. After the extraction and clean up, a known volume of extract is concentrated under a gentle stream of nitrogen to 0.5 mL.

The resulting extracts are vialed and fortified with labeled PAH internal standard compounds prior to analysis by gas chromatography with a 95% dimethylpolysiloxane (DB-5) column and a mass spectrometer detector in SIM Mode.

Primary Reference

Environment Canada Method ESTD-OR-20: Determination of Total Petroleum Hydrocarbons,n-Alkanes, Polycyclic Aromatic Hydrocarbons and Selected Biomarkers in Oil and Petroleum Products.
EPA SW-846 Method 8270D: Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry (GC/MS), Rev. 4, February 2007.

METHOD SUMMARY

BQ-GFPC-LSC: Radionuclides by Gas-Flow Counting Proportional Counting

BQL SOP-00008

Introduction

This determination of radionuclides by gas-flow proportional counting has been developed by Maxxam Analytics International Corporation; Kitimat as a proprietary method based on standard industry practices and the radiological and chemical properties of specific analytes.

When most radioactive nuclei decay, either a beta particle or an alpha particle is emitted. When these ionized particles strike gas molecules, electric charge separation occurs. In Gas Flow Proportional Counting, these charges are collected under a high voltage and the resulting current pulses counted to indicate the number of ionized particles emitted and thus the intensity of the radioactivity. Because the pulses due to alpha particles are much larger than those due to beta particles one can discriminate between the two types of radiation.

For the determination of individual radioisotopes, radioisotope tracers or spiked samples are used to monitor the chemical yield of the separation and source preparation processes.

The sources are presented to a gas-flow proportional detector for a period of time and counts are collected in separate alpha and beta particle channels. The channel counts are used to determine the amounts of the isotopes of interest in the sources and therefore in the samples processed.

Accuracy and precision of the results are a function of the mass and radiochemical purity of the sources prepared from the samples, the counting time, and the detector efficiency and background.

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Determination of Phosphorus (all forms) in Waters by Colorimetry (FIA)

CAM SOP-00407

Reference Methods:

Total and Hydrolyzable P: Standard Methods 22nd Ed. Method 4500-P H (Manual Digestion and Flow Injection Analysis for Total Phosphorus)

Ortho-Phosphate: Standard Methods 22nd Ed. Method 4500-P G (Flow Injection Analysis for Ortho-Phosphate)

Digestion: Standard Methods 22nd Ed. Method 4500-P B Sample Preparation

Scientific Principle

The colorimetric method is based on specific reaction of the orthophosphate ion. Dissolved Orthophosphate (DOP) reacts with antimonyl molybdate reagent (in an acid medium) to form the antimonyl-substituted 12-molybdo-phosphoric acid icosahedral cage structure. This complex is reduced with ascorbic acid to yield an intense blue chromophore proportional in concentration to the orthophosphates present in the original sample. The absorption is measured at 880 nm. Polyphosphates are converted to the orthophosphates forms by sulphuric acid digestion and organic phosphorus is converted to orthophosphates by persulphate digestion.

$$2H_2SO_4 + \text{organic phosphorous} + 3K_2S_2O_8 \rightarrow 2K_3PO_4 + 8H_2SO_4 + xH_2O + xCO_2$$

The intense blue chromophore produced, which is proportional to phosphorous concentration, obeys Beer's Law.

Beer's Law

$$A = a * b * c$$

Where: A = absorbance
a = analyte molar absorptivity co-efficient
b = path length
c = concentration of analyte

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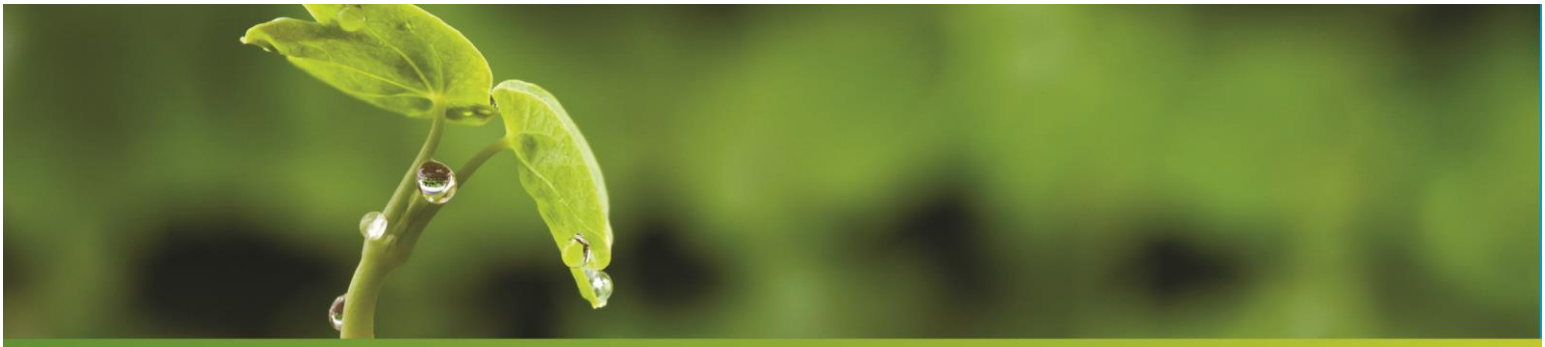
This analytical method detects orthophosphate only. Samples for total phosphorus are digested with sulphuric acid and potassium persulphate. This digest converts organic phosphorus compounds and polyphosphates to orthophosphate. Samples treated this way are reported in mg/L as Total P. Unpreserved samples may be filtered through 0.45um filter prior to digestion. Omission of persulphate ($K_2S_2O_8$) in digestion will result in low or no recovery of organic phosphorus, but will convert polyphosphates to orthophosphate. This is acid hydrolyzable P. If samples are analyzed without digestion, only orthophosphate will be detected. In this way, phosphorus forms can be distinguished. Results for filtered samples are reported as mg/L dissolved P.

- Orthophosphate (OrthoP): Analysis of unpreserved sample without digestion.
- Acid Hydrolyzable Phosphorus (HydroP): Analysis of unpreserved sample after sulphuric acid digestion. (skip persulphate addition)
- Total Phosphorus (TP): Analysis of sample after sulphuric/persulphate digestion.
- Organic phosphorus can be calculated as the difference between TP analysis and HydroP analysis.

Scope

This method is used for surface waters, ground waters, drinking waters, liquid sewage and industrial samples plus MISA samples (Municipal and Industrial Strategy for Abatement). Method is not applicable to soils, sludge, oil, or other solid material. This method can be used for the determination of orthophosphate (OrthoP), ortho + polyphosphates (HydroP), and total phosphorus. All results are reported as mg/L P. Organic phosphorus and polyphosphates can be determined by calculation.

All forms can be determined in filtered or unfiltered samples. If done on filtered samples, results are reported as “dissolved” or “filtered”.



METHOD SUMMARY

Ammonia in Waters Biosolids and Soil Samples by Colourimetry

CAM SOP-00441

Reference Method: USGS I-2522-90 Nitrogen, Ammonia, Colourimetry, Salicylate-Hypochlorite, Automated-Segmented Flow

Soil/ Biosolid Preparation: Soil Sampling and Methods of Analysis, M.R. Carter, 1993

Scientific Principal

Ammonia reacts with salicylate and hypochlorite ions in the presence of a nitroprusside catalyst in a phosphate buffer to form the salicylic acid analog of indophenol blue. The resulting color is proportional to the ammonia concentration. The ammonia nitrogen is measured colorimetrically at 660 nm, using Quikchem 8000 by Lachat Instrument. Ammonia and ammonium ions are initially buffered to pH10 to convert ammonium cation to ammonia before being analyzed and the total ammonia (the sum of NH_4^+ + NH_3) is reported as mg/L Nitrogen. The indophenol blue colour produced, which is proportional to ammonia concentration, obeys Beer's Law.

In aqueous solution, unionized ammonia (NH_3) exists in equilibrium with ammonium ion and hydroxide ion. The equilibrium constant for this reaction is a function of temperature and solution pH. Thus, if the equilibrium constant is known for a particular temperature and the pH of the solution is also known, the fraction of un-ionized ammonia can be calculated. Then, if the total ammonium concentration is known from laboratory analysis, the un-ionized ammonia concentration can be calculated. Unionized ammonia is calculated using field temperature, field pH and lab ammonia nitrogen determination.

Applicability

This procedure is applicable to the analysis of potable and non-potable waters including surface and ground waters, saline waters and domestic and industrial wastewaters. It can also be used for the analysis of soils/sediments and biosolid (sludge) samples after an appropriate extraction procedure.

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Chlorophyll A and Pheophytin

Samples are collected by filtration onto Whatmann GF/F glass-fibre filters at low pressure and under low light. Pigments are extracted in 90% (w/w) acetone in a freezer for 24 hours. After extraction, the solvent is warmed to room temperature and fluorescence read in two Turner Model 10 Series fluorometers. The first is configured for the acidification method (based on methods developed by Holm-Hansen et al. and Lorenzen et al; see EPA Method 445), in which fluorescence is measured before and after acidification with 10% HCl to correct for the contributions of chlorophyll degradation products (phaeopigments). The second is configured for the non-acidification method (Welschmeyer 1994), which is less sensitive to interference from chlorophyll b and chlorophyll degradation products. Both fluorometers are calibrated with purified chlorophyll a (Sigma C6144) and a solid standard is measured with each use to check for instrument drift. References are below!

Calculation

Acidification Technique

Chlorophyll a (µg/l) = F(Fb – Fa) / (ml filtered)(sensitivity)

Pheopigment (µg Chl eq/l) = F((A*Fa) – Fb) / (ml filtered)(sensitivity)

Where:

F = Calibration factor
A = Acid ratio of fluorometer
Fb = Voltage before acidification, corrected for blank at that sensitivity
Fa = Voltage after acidification, corrected for blank at that sensitivity

Note: Pheopigment is expressed as µg chlorophyll equivalents per liter. That is, if 1 µg/l of chlorophyll a is acidified, the resulting concentration of pheophorbide is 1µg chlorophyll equivalents per liter. The actual concentration is 0.663 µg/l.

Welschmeyer Technique

Chlorophyll a (µg/l) = (F*Fb) / (ml filtered)(sensitivity)

Where:

F = Calibration factor
Fb = Voltage before acidification, corrected for blank at that sensitivity.

Holm-Hansen O (1978) Chlorophyll *a* determination: improvements in methodology. Oikos 30:438-447
Holm-Hansen O, Lorenzen CJ, Holmes RW, Strickland JDH (1965) Fluorometric determination of chlorophyll. J Conseil 30:3-15
Lorenzen CJ, Jeffrey SW (1980) Determination of chlorophyll in sea water. UNESCO Reports Technical Papers in Marine Science Vol. 35 pp 1-20
Lorenzen CJ, Newton Downs J (1986) The specific absorption coefficient of chlorophyllide *a* and phaeophorbide *a* in 90% acetone, and comments on the fluorometric determination of chlorophyll and pheopigments. Limnol Oceanogr 31:449-452
Welschmeyer NA (1994) Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and phaeopigments. Limnol Oceanogr 39:1985-1992

Digestion of Aqueous Samples for Metals by ICPMS or ICP-OES Analysis BBY7SOP-00003

Reference Method: Standard Methods for the Examination of Water and Wastewater. 22nd Edition, 2012. Rice, E.W., Baird, R. B., Eaton, A.D., & Clesceri, L.S., eds. American Public Health Association, American Water Works Association, & Water Environment Federation, 3030 F. Nitric Acid-Hydrochloric Acid Digestion.

SECTION 1: Purpose/Principle of Method

Aqueous samples containing varying amounts of particulate matter are digested to:

(1) Solubilize trace metals that are either contained in or adhered to the particulate;

(2) Stabilize the metal species and ensure reproducible results in the digestate;

(3) Remove the organics by oxidation and volatilization.

The completed digestions are submitted to the metals instrumentation laboratories for analysis by Inductively Coupled Plasma Mass Spectroscopy (ICPMS) or by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

SECTION 2: Scope

This method is applicable to aqueous samples requiring total metals analysis. If a sample is very turbid, further instructions need to be followed. Dilute the sample prior to digestion may be required. The completed digests are submitted to metals instrumentation personnel for metals analysis by ICPMS or ICP-OES.

SECTION 3: Detection Limits

Not applicable to this method.

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SECTION 4: Interferences

Interferences for this procedure are most often related to the instrumentation that performs the analysis on the digested solutions. These interferences are typically due to the matrix (i.e. seawater, high suspended solids, colour) or high metal concentrations of the samples being analysed.

SECTION 5: Sample Handling & Preservation

Matrix	Sample Container	Holding Time	Storage Conditions	Preservation
Water (Total or Dissolved Metals)	Pre-cleaned (metals-free) polyethylene bottle.	Preserved samples: 6 months except 28 days for Hg	Room temperature	Field filtered, if possible. **Field preservation** 1 mL 1:1 HNO_3:RODI water per 120 mL sample. **In lab preservation** 0.5 ml HCl and 0.5 ml conc. HNO3 per 100 ml sample is added to field-preserved samples. Alkaline samples may require additional preservative to achieve pH <2.
		Unpreserved samples: Max 24 hours		**In lab preservation** 1 mL conc. HNO_3 and 0.5 ml con. HCL per 100 mL sample. Alkaline samples may require additional preservative to achieve pH <2.

METHOD SUMMARY

Extraction of Metals in High Salt Content Samples

BBY7SOP-00007

Reference Methods:

EPA Method 200.10, Determination of Trace Elements in Marine Waters by On-Line Chelation Preconcentration and Inductively Coupled Plasma – Mass Spectrometry, Revision 1.4, April 1991

Elemental Scientific M-SFMG-62 SC-DX seaFAST Method Guide, Agilent 7500/7700

CETAC Technologies, "Use of the SPR-IDA Reagent for the Determination of Trace Metals in a Coastal Seawater Reference Material".

SECTION 1: Purpose/Principle of Method

This method describes the extraction and pre-concentration of trace elements in marine waters, including estuarine waters, seawater and brines, using an iminodiacetate-functionalized chelating resin in preparation for analysis by ICP-MS.

Buffering and chelation may be performed manually using a chelating suspended particulate reagent before introduction into the ICP-MS or may be automated using a chelation column in an automated sample introduction system.

SECTION 2: Scope

This method is applicable for the extraction and pre-concentration of the following elements in marine waters, including estuarine waters, seawater and brines:

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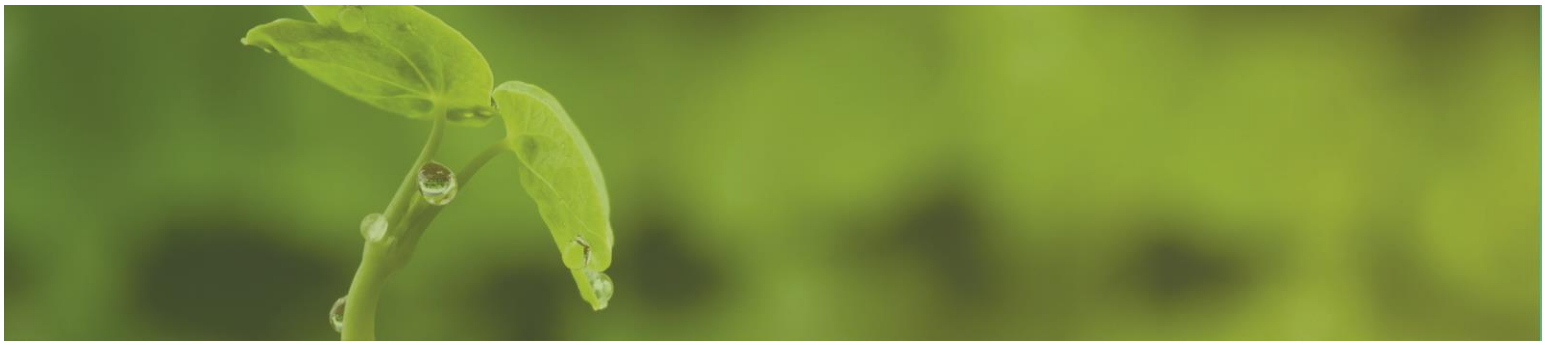


Cd, Cu, Co, Fe, Mn, Ni, Pb, U, and Zn by manual chelation extraction;

Al, Cd, Co, Cu, Fe, Pb, Mn, Ni, Ti, V, U, Zn by automated chelation extraction.

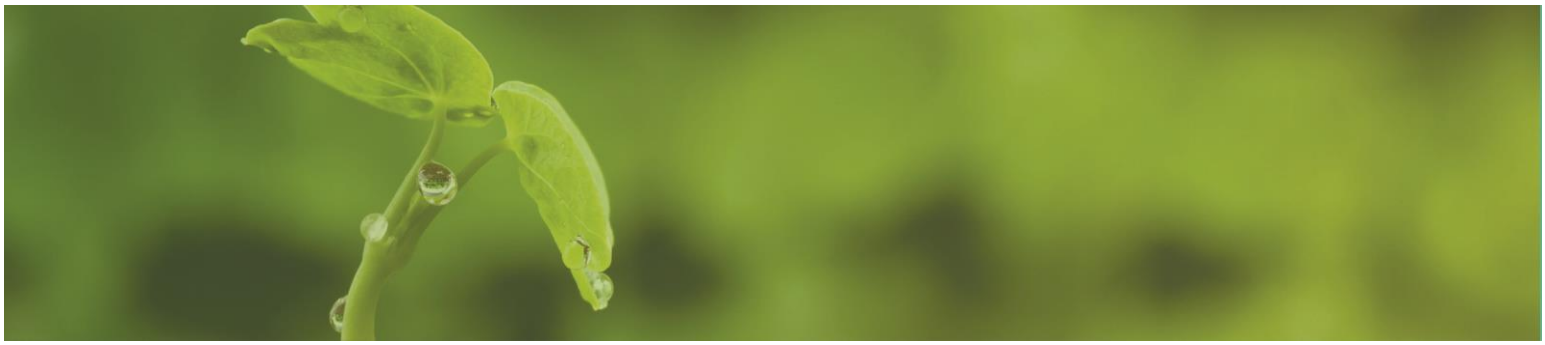
This method is applicable to filtered water samples (for dissolved metals) and unfiltered and undigested water samples (for total metals).

SECTION 3: Detection Limits

Element	Manual Chelation (SPR-IDA) METDC-SE METTUC-SE (µg/L)	Automated Chelation METDSFC-SE METTSFC-SE (µg/L)
Aluminum (Al)	N/A	0.5
Cadmium (Cd)	0.01	0.001
Cobalt (Co)	0.05	0.01
Copper (Cu)	0.05	0.005
Iron (Fe)	1	0.5
Lead (Pb)	0.05	0.001
Manganese (Mn)	0.2	0.005
Nickel (Ni)	0.05	0.01
Titanium (Ti)	N/A	0.5
Vanadium (V)	N/A	0.01
Uranium (U)	0.01	0.001
Zinc (Zn)	0.2	0.05

SECTION 4: Interferences

This method provides selective elimination of species giving rise to polyatomic spectral interferences on certain transition metals (e.g. removal of the chloride interference on vanadium). As the majority

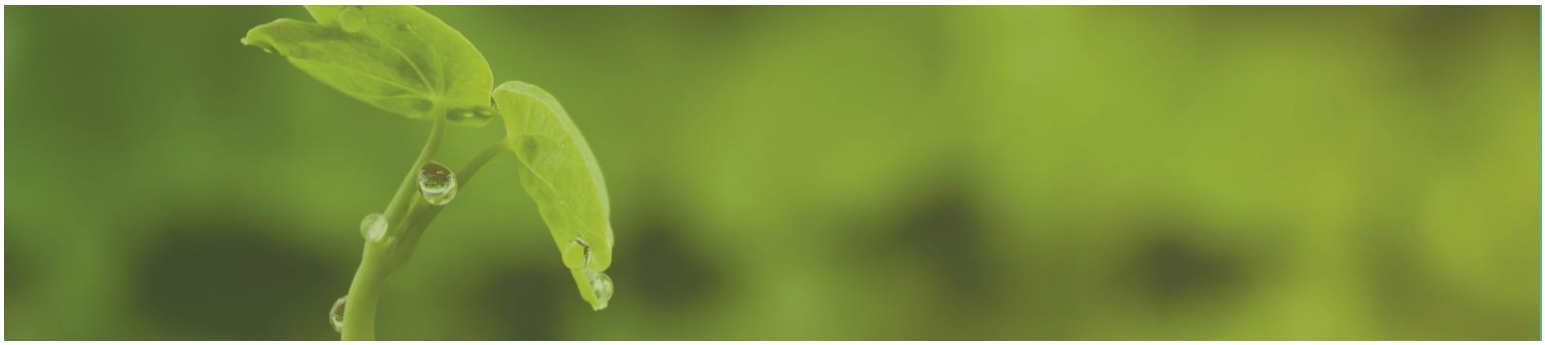


of the sample matrix is removed, matrix-induced physical interferences are also substantially reduced.

Low recoveries may be encountered in the preconcentration cycle if the trace elements are complexed by competing chelators in the sample or are present as colloidal material. Acid solubilization pretreatment is employed to improve analyte recovery and to minimize adsorption, hydrolysis and precipitation effects.

Memory interferences from the chelating system may be encountered especially after analyzing a sample containing high concentrations of the analytes. A thorough column rinsing sequence following elution of the analytes is necessary to minimize such interferences.

Sample contamination resulting in falsely elevated target analyte values may occur in a variety of ways. Extreme care must be taken in the cleanliness of all apparatus, reagents and analytical techniques.



SECTION 5: Sample Handling & Preservation

Sample Matrix	Sample Container	Minimum Volume	Holding Time	Storage Conditions	Preservation
Dissolved Metals in Waters	120 mL HDPE	100 mL	6 months after preservation	Room Temperature	Field filtered, if possible then preserve with 1 mL of 1:1 HNO_3:DI Water per 120 mL sample.
Total Metals in Waters	120 mL HDPE	100 mL	6 months after preservation	Room Temperature	1 mL of 1:1 HNO_3:DI Water per 120 mL sample. Highly alkaline samples may require additional preservative if pH is not <2.

METHOD SUMMARY

Mercury in Water

SOP #: ATL SOP-00026

Reference: USEPA Method 245.1.

1. Scope and Application

This method is designed for the digestion and analysis of total mercury in water and aqueous samples as referenced in EPA Method 245.1. The Reporting Detection Limit (RDL) for this procedure is <0.013 ug/L.

2. Summary of Method

A 30 mL aliquot of preserved sample is digested at 95°C with a mixture of sulphuric acid, nitric acid and an excess of potassium permanganate, along with potassium persulphate. Following digestion, excess potassium permanganate is destroyed with hydroxylamine hydrochloride and samples are analyzed for mercury using an automated CVAAS Analyzer. During analysis, the digested samples are mixed with stannous chloride which reduces the mercury to its atomic state. The mercury is sparged from solution using nitrogen gas and the vapour is swept into the absorption cell. The instrument signal (absorbance) at 253.7 nm is proportional to the concentration of mercury in the sample.

3. Quality Assurance

Second source reference materials, method blanks, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of 1 for every 20 samples.

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METHOD SUMMARY

Total/Dissolved Organic Carbon

SOP #: ATL SOP-00037

Reference: APHA Standard Methods, 5310C Persulfate – Ultraviolet Oxidation Method

1. Scope and Application

This method is applicable for the analysis of Total Organic Carbon (TOC) in surface waters, ground waters and other aqueous environmental samples. These same matrices can also be analyzed for Dissolved Organic Carbon (DOC) after the samples have been passed through a 0.45-micron filter. The Reporting Detection Limit (RDL) is 0.5 mg/L.

2. Summary of Method

The sample (samples for DOC analysis are filtered in order to remove particulate matter) is acidified and then sparged with nitrogen in order to remove all inorganic carbon in the form of carbon dioxide. Following the addition of a digestion reagent, the sparged sample is then oxidized using UV radiation. The resulting carbon dioxide is dialyzed and reacted with a weak buffered phenolphthalein solution. The decrease in colour (measured by Technicon AutoAnalyzer @ 550 nm) is directly proportional to the concentration of organic carbon.

3. Quality Assurance

Second source blank spikes, method blanks, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of 5%.

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METHOD SUMMARY

Total Suspended Solids in Water and Seawater

SOP #: ATL SOP-00007

Reference: EPA Method 160.2.

1. Scope and Application

This method is applicable to surface, ground, and saline waters as well as to domestic and industrial wastes. The practical range of the determination is 0.5 - 100,000 mg/L. The Reporting Detection Limit (RDL) is 0.5 mg/L based on a sample volume of 1000 mL.

2. Summary of Method

A known quantity of sample is vacuum filtered through a standard pre-weighed glass fiber filter. The filter plus retained solids is oven dried, cooled in a desiccator and weighed. The increase in weight from the original filter weight represents the total suspended solids present.

3. Quality Assurance

A minimum of one Method Blank, one Internal Reference Material and one Sample Duplicate is analyzed with each batch of samples, with a minimum QC frequency of 5 %.

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METHOD SUMMARY

Nitrogen (Ammonia) in Aqueous Solutions by Konelab using Phenate Method

BBY6SOP-00009

Reference Method: Standard Methods for the Examination of Water and Wastewater, 2012. Rice, E.W., Baird, R. B., Eaton, A.D., & Clesceri, L.S., eds. American Public Health Association, American Water Works Association, & Water Environment Federation, 22nd Edition. Method 4500-NH3− G, Nitrogen (Ammonia) Automated Phenate Method

SECTION 1: Purpose/Principle of Method

Alkaline phenol and hypochlorite react with ammonia to form indophenol blue that is proportional to the ammonia concentration. The blue colour formed is intensified with sodium nitroprusside.

Ammonia is determined colourimetrically using the Berthelot reaction (Indophenol Blue Reaction) in the presence of EDTA under basic conditions. The Berthelot reaction is a three-stage process. In the first stage, the sample pH is adjusted to basic conditions to convert ammonium to ammonia. The ammonia then undergoes a chlorination reaction with hypochlorite to form a monochloramine. In the second stage, the monochloramine is further reacted with alkaline phenol in the presence of nitroprusside to form quinonechloramine. In the third stage, the quinonechloramine reacts further with phenol to form the blue indophenol complex. The indophenol formed is proportional to the ammonia concentration and is measured colourimetrically at 660 nm.

The analysis is conducted in a range within which Beer's law is obeyed.

$$A = \varepsilon c l$$

Where: A= absorbance

ε = molar absorptivity (mg/L)-1cm-1

c = concentration of the absorbing species (mg/L)

l = path length (cm)

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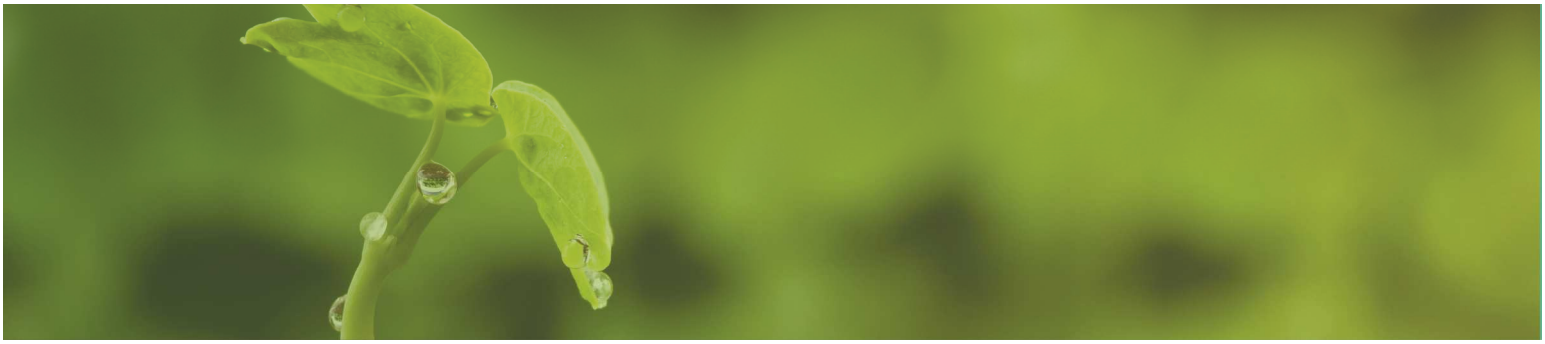
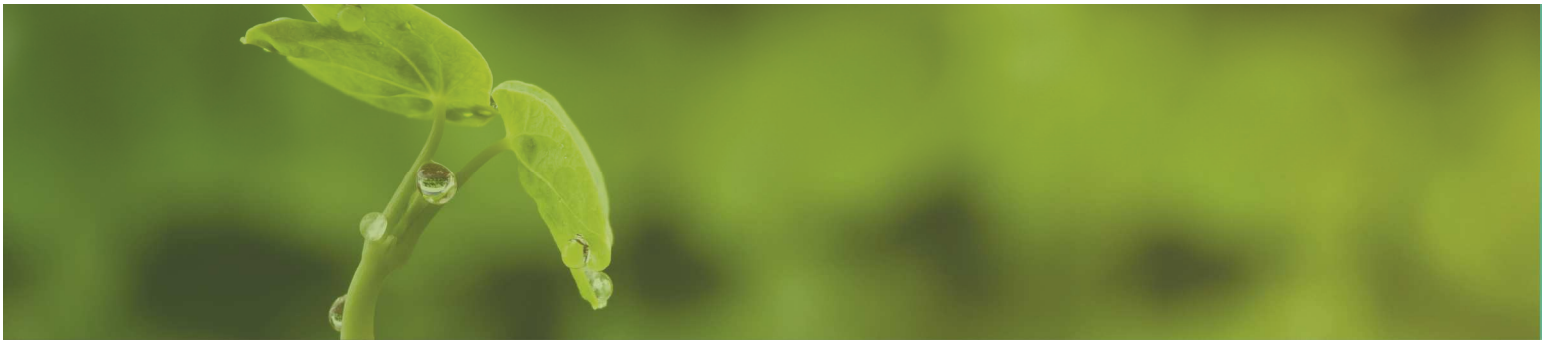


SECTION 2: Scope

This method is applicable to the determination of ammonia nitrogen in drinking waters, surface waters, groundwaters, saline waters, precipitation samples, and domestic and industrial wastes. This method is also applicable to determination of soluble ammonia nitrogen in aqueous extracts soil and solid materials.

SECTION 3: Detection Limits

Parameters	RDL and Range
Ammonia N (Leachable)	0.0050 – 1.00 mg/L NH_3 as N
Ammonia Nitrogen-Unpreserved	0.0050 – 1.00 mg/L NH_3 as N
Ammonium-Acid Rain Samples	0.25 – 50 mg/kg NH_3 as N
Soluble Ammonia Nitrogen-1:5 DI Leach	0.50 – 100 mg/kg NH_3 as N
Ammonia-N Dissolved Unfiltered/Unpreserved	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N Dissolved	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N (Preserved)	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N - Regular Columns 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N - Humidity Cell 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N HCT Residue 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N MEND SFE Extract 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N MWMP Extract 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N



Parameters	RDL and Range
Ammonia-N NAG Extract 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N - SAD Columns 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N
Ammonia-N SPLP Extract 0.005 mg/L	0.0050 – 1.00 mg/L NH_3 as N

SECTION 4: Interferences

Seawater contains calcium and magnesium ions in sufficient concentrations to cause precipitation problems during analysis. Adding EDTA and sodium potassium tartrate reduces the problem.

Eliminate any marked variation in acidity or alkalinity among samples because intensity of colour is pH-dependent. Samples are neutralized eliminating pH variability.

Ensure the pH of wash water and standard ammonia solutions approximates that of the sample, e.g. match sample preservation in wash water and standards.

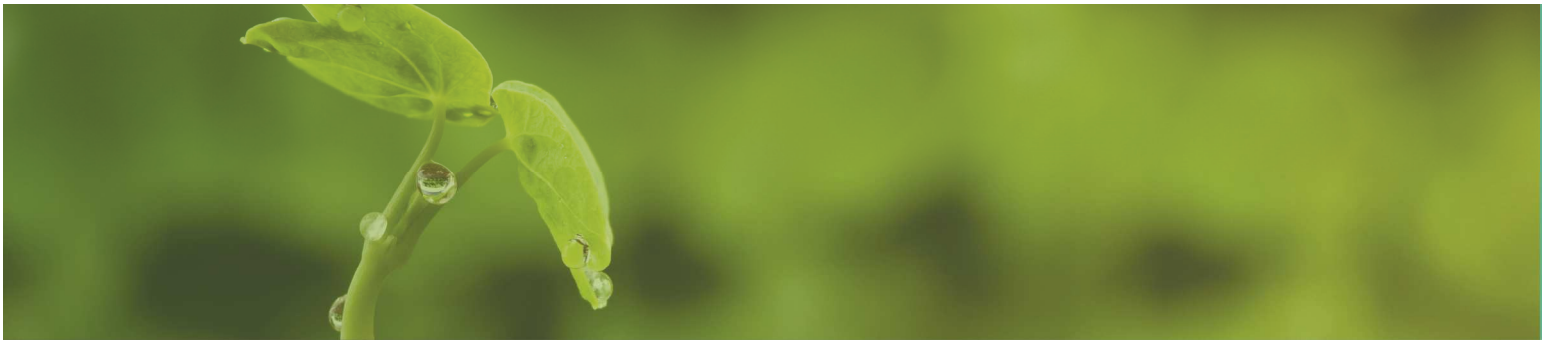
Remove interfering turbidity by filtration.

Colour in the samples that absorbs in the photometric range used for analysis interferes.

Residual chlorine can react with ammonia. If residual chlorine is present, the sample must be treated with sodium thiosulphate at the time of collection.

If hydrogen sulphide is present, remove by aerating vigorously until sulphide odour no longer can be detected.

Acidification produces interferences when exchangeable ammonium is present in unfiltered solids.



SECTION 5: Sample Handling & Preservation

Sample Matrix	Sample Container	Minimum Volume	Holding Time	Storage Conditions	Preservation
Water	120 mL HDPE bottle or equivalent	25 mL	3 days from sampling time or 28 days if preserved	1-6°C	Total: 1 mL 1:1 H_2SO_4 per 120 mL sample Dissolved: Filter through 0.45 µm filter then add 1 mL 1:1 H_2SO_4 per 120 mL sample
Soil extracts	50 mL Falcon Tube	25 mL	3 days from extraction	1-6°C	None

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ENVIRONMENTAL SERVICES

ANALYSIS OF RADIOACTIVITY IN WATER

Introduction

Health Canada Guidelines

Special Guidelines for Ontario

Logistics

The Maxxam Advantage

Radioactivity can occur naturally in water due to dissolved uranium, thorium and their daughter isotopes. Health Canada's Guidelines for Canadian Drinking Water Quality (2014) lists six radionuclides that are commonly detected in water. Health Canada has established maximum acceptable concentrations in drinking water for two natural and four artificial radionuclides and for total uranium in chemical form (analyzed using Maxxam's standard analysis for metals).

The radionuclides most commonly detected in our radiological analysis of Canadian drinking water are:

- Natural: Lead-210 (^{210}Pb), Radium-226 (^{226}Ra)
- Artificial: Tritium, Strontium-90(^{90}Sr), Iodine-131 (^{131}I), Cesium-137 (^{137}Cs)

Health Canada Guidelines

Maxxam performs radiological testing of water samples for all provinces and territories following federal guidelines from Health Canada. Our analysis can be used to evaluate potential health risks associated with water quality. The results provide a starting point to assess treatment system requirements and costs. Health Canada's "Guidelines for Canadian Drinking Water Quality: Guideline Technical Document - Radiological Parameters"[1] lists the Maximum Acceptable Concentrations (MAC) for radionuclides. There are special requirements for Ontario which are summarized in a separate section below.

Maxxam recommends an initial screening for radioactivity by measuring gross alpha and gross beta activity. Compliance with Health Canada's Guidelines may be inferred if the results for gross alpha and gross beta activity are less than 0.5 Bq/L and 1 Bq/L, respectively (^{226}Ra for alpha activity; ^{90}Sr for beta activity). Maxxam's detection limits are below these levels unless the drinking water contains >300 mg/L of dissolved solids. When the gross alpha activity concentration exceeds 0.5 Bq/L or the gross beta activity concentration exceeds 1 Bq/L, Maxxam will make recommendations for further sampling, identification and quantification of the specific radionuclides that are at the source of the elevated results.

Special Guidelines for Ontario

Maxxam is the only commercial laboratory licensed by the Ontario Ministry of the Environment (MOE) for radionuclide analysis on drinking water samples. Ontario drinking water samples require special handling. A special submission form is required for the analysis of water samples for human consumption in Ontario (unless the water supply is federally regulated).

ANALYSIS OF RADIOACTIVITY IN WATER

"We recommend an inexpensive, initial screening for radioactivity by determining the gross alpha and gross beta activity. This shows the level of radioactivity in the water but not the specific radioisotopes. Health Canada suggests that compliance with the guidelines may be inferred if the measurements for gross alpha and gross beta activity are less than 0.5 Bq/L and 1 Bq/L, respectively."

– Steven Simpson, B.Sc./MBA
Lab Director

Maxxam is the Canadian market leader in analytical services and solutions to the energy, environmental, food and DNA industries and a member of the Bureau Veritas Group of companies – a world leader in testing, inspection and certification services. We support critical decisions made by our customers through the application of rigorous science and the knowledge and expertise of our over 2500 employees.

For more information please contact:
Email: rad@maxxam.ca or
Call us toll-free: 1 877 726 3080

Maxxam provides measurements of the over 70 radionuclides listed in the Ontario Safe Drinking Water Act. Specific regulations vary by municipality, but we typically recommend starting with the "Ontario Radionuclide Screen" for gross alpha, gross beta and Tritium. We also offer the "Ontario MOE Certificate of Approval Analysis package". Both services are offered to comply with O.Reg 170/03 and require the submission of a Chain of Custody document BQL FCD-00080 "Drinking Water Chain of Custody Record for Radiological Parameters".(2)

Logistics

All water samples for radioactivity analysis are processed by Maxxam's laboratory in Mississauga, Ontario. Standard turn-around-time (TAT) is 10 working days (2 weeks) from date of receipt at the lab. The turn-around-time for ^{210}Pb is typically 3 weeks and the TAT for multiple isotopes is 3-4 weeks.

The Maxxam Advantage

Experience: Test protocols are conducted using proven technologies operated by our highly experienced staff of scientists and technicians. With over 30 years of experience, Maxxam's experts in radiological analysis can provide advice on applying the appropriate analytical method(s) to meet your requirements. Upon request, we can also customize analysis packages to meet your objectives.

Quality: Maxxam has a strong Quality Management System (QMS) which encompasses both quality assurance and quality control. More than 30 people are employed on Maxxam's Quality Assurance (QA) team as Regional Managers, Specialists and Coordinators. QA staff are responsible for carrying out the monitoring, documentation and training required by the company's QMS. To ensure independence, integrity and effectiveness of their functions, these employees report to the National Director of Quality, who reports directly to Maxxam's CEO.

NOTES:

(1) http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/radiological_para-radiologiques/index-eng.php

(2) http://maxxam.ca/resources/chain-of-custody-coc-forms

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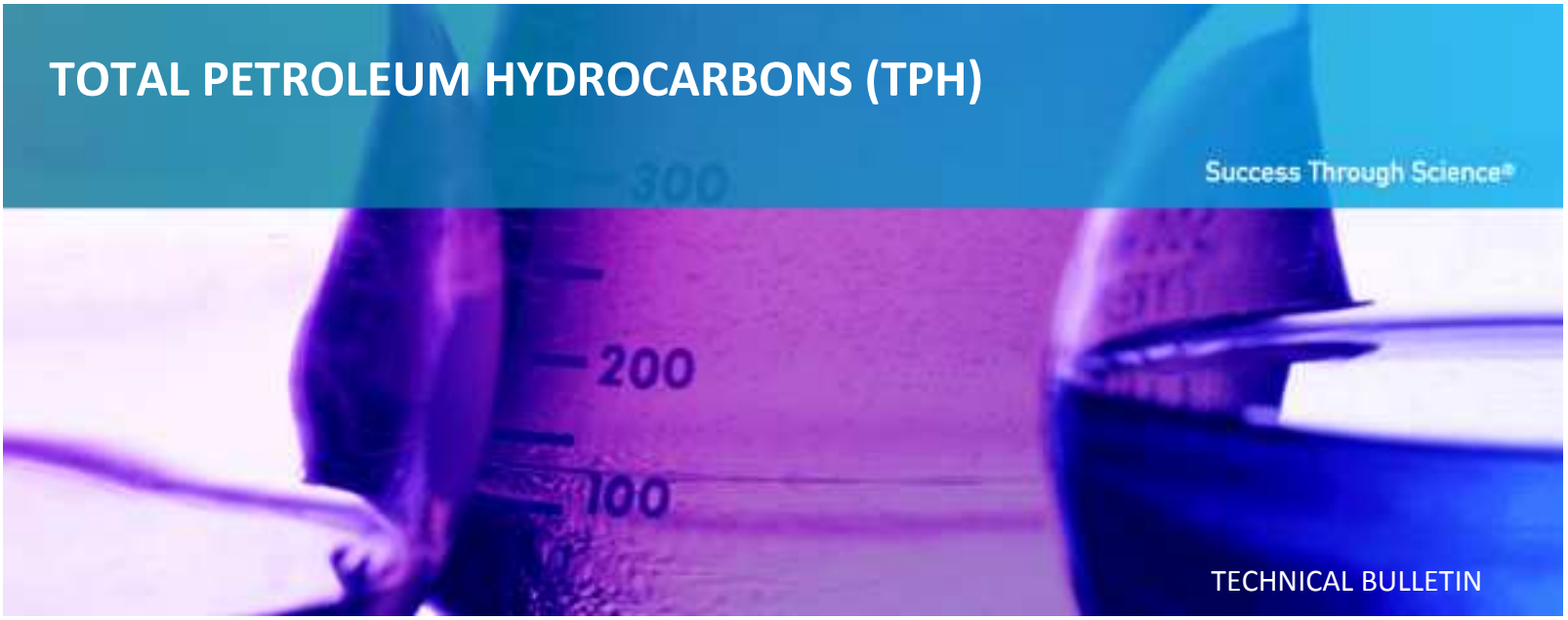
TOTAL PETROLEUM HYDROCARBONS (TPH)



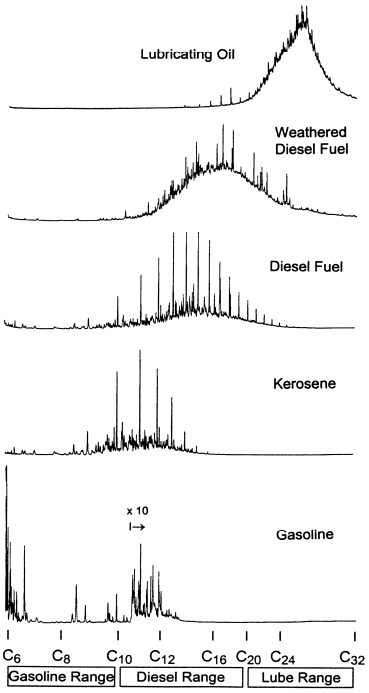


Figure 1: Petroleum hydrocarbon gas chromatograms.

Petroleum hydrocarbons are among the most commonly used chemicals in the world. There is considerable potential for contamination of the environment during their manufacture, transportation, storage and disposal. These products can be found in soil, sediment, water and biota.

The Atlantic Provinces have established remediation criteria for petroleum hydrocarbons using a Risk Based Corrective Action (RBCA) approach. ([1]) The clean up guidelines depend on the sensitivity of the site and the nature of the product. For more detailed information, visit http://www.atlanticrbca.com.

Analysis

1) **Benzene, Toluene, Ethylbenzene, Xylene(s) (BTEX) and Gasoline Range Organics (C_6 - C_{10}):** Samples are analyzed by either purge and trap – gas chromatography / mass spectrometry or headspace – gas chromatography (MS / flame ionization detectors). A surrogate compound is added to samples to monitor the efficiency of the preparation and analysis steps. Water samples are analyzed directly. Soil samples are initially extracted with methanol. This extract is then diluted into water prior to analysis. BTEX and gasoline range organics are quantified using a reference mixture of aliphatic and aromatic compounds.

2) **Extractible Hydrocarbons – Diesel (>C_{10} – C_{16}), Diesel (>C_{16} – C_{21}) and Lube (>C_{21} - C_{32}) Range Organics:** Water and soil samples are extracted with organic solvent(s). Two surrogate compounds are added to the samples prior to extraction. Water sample are tumbled with the solvent. Soil samples are shaken vigorously with a solvent mixture. Biota samples are extracted using an organic solvent, after a homogenization step. The solvent extracts are analyzed using capillary column gas chromatography (flame ionization detector). Diesel and lube range organics are quantified using a reference mixture of aliphatic and aromatic compounds.

Note: The gasoline signal >C_{10} shown in Figure 1 has been amplified 10 times to illustrate that gasoline components elute within the diesel range.

Detection Limits

Detection limits are evaluated using U.S. EPA protocols. Reporting Detection Limits (RDLs) are as follows:

Component	Soil (mg/kg)
Benzene	0.025*
Toluene	0.025
Ethylbenzene	0.025*
Xylene(s)	0.05
Gasoline Range (C_6-C_{10})	2.5
Diesel Range (>C_{10}-C_{16})	10
Diesel Range (>C_{16}-C_{21})	10
Lube Range (>C_{21}-<C_{32})	15

Component	Water(mg/L)
Benzene	0.001
Toluene	0.001
Ethylbenzene	0.001
Xylene(s)	0.002
Gasoline Range (C_6-C_{10})	0.01
Diesel Range (>C_{10}-C_{16})	0.05
Diesel range (>C_{16}-C_{21})	0.05
Lube Range (>C_{21}-<C_{32})	0.1

*Low-level soil RDLs required for PIRI Tier 1 / potable water circumstances are 0.005 mg/kg for Benzene and 0.1 mg/kg for Ethylbenzene.

Quality Assurance

A method blank, spiked blanks, matrix spike and a replicate sample are analyzed with each batch of twenty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

Sampling

Water samples are collected in 3 x 40 mL purge and trap vials and 2 x 250 mL glass bottles. The 40 mL purge and trap vials as well as the 250 mL bottles are preserved with sodium bisulfate. Because volatile compounds can transfer into any air remaining in the vial, the 40 mL purge and trap vials should be completely filled, leaving no headspace (air bubbles). Turn the vial upside down to check for bubbles. If bubbles are present, the sample should be recollected. Note: to avoid sample breakage, the 250 mL bottles should have a small quantity of headspace to allow for sample expansion. Sodium bisulfate helps prevent bacterial degradation of organic compounds (e.g. BTEX). Containers require Teflon lined caps. Sufficient soil or sediment should be collected to fill a 250 mL and a 60 mL glass soil jar with a minimal amount of headspace. Soil samples for BTEX and gasoline are methanol preserved upon receipt in the lab. Samples should be packed in ice during shipping to maintain a temperature of <10°C (<4°C optimal) and delivered as soon as possible to the laboratory. Sampling containers with preservatives are available at no charge from Client Services upon request.

Hydrocarbon Fingerprinting

Hydrocarbon "fingerprinting" involves identifying the nature of the petroleum product through careful evaluation of the gas chromatograms. Examples are provided in Figure 1 of various petroleum products represented chromatographically. TPH weathering occurs in the natural environment as a result of water solubilisation, evaporation and oxidation. These processes produce changes in the chromatograms. The resemblance comment on the analytical report is provided for general guidance on the nature of the hydrocarbon contaminants. Weathering is based on C17 : pristine and C18 : phytane ratios.

1) Reference: "Atlantic RBCA Guidelines for Laboratories", Version 3.0, 2010.

Maxxam is an analytical services and solutions company that is passionately committed to delivering exceptional science through outstanding service. We set ourselves apart by continually contributing to our customers' success through the application of our knowledge and expertise. We are dedicated to success with responsibility - to our stakeholders, to our communities, and to the environment.

APPENDIX D

Commercial Fish Methods

METHOD SUMMARY

Total Extractable Hydrocarbons (>C10-C32) in Fish and Shellfish

SOP #: ATL SOP-00112

Reference: Atlantic PIRI Guidelines for Laboratories

1. Scope and Application

This method is designed for the extraction and analysis of petroleum hydrocarbons, including diesel range organics (>C10-C21) and lubricating oils (>C21-C32) in tissues and biota. The Reporting Detection Limits (RDLs) are as follows: >C10-C21 (15 mg/kg) and >C21- C32 (15 mg/kg) and are on a wet weight basis.

2. Summary of Method

The tissue is homogenized in blender and a 5 g portion is weighed out and spiked with a surrogate compound (n-dotriacontane). This compound is used to monitor the efficiency of the sample preparation steps. The sample is extracted with a 50/50 acetone/hexane solvent using an accelerated mechanical extraction (AME) process. An aliquot of the extract is removed and interfering compounds are eliminated using a silica gel column clean-up procedure. The extract is then vialed and analyzed by gas chromatography with flame ionization detection. The GC-FID system is calibrated with multi-component standards of known concentration which elute in the >C10-C32 range. A calibration curve is generated for each carbon range (>C10-C21 and >C21-C32) using the response factor of the compounds that elute in each range. Calibration accuracy is verified by analyzing an independent reference standard. The day-to-day stability of the calibration is confirmed by analyzing calibration check solutions with each batch of samples. Sample products are identified by comparing each product to a library of reference products.

3. Quality Assurance

Method blanks (containing commercially available fish tissue), spiked blanks (transformer oil spiked on commercially available fish tissue), matrix spikes (transformer oil spiked onto tissue samples) and replicate samples are analyzed with each batch of twenty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

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METHOD SUMMARY

Mercury in Biota Samples

SOP #: ATL SOP-00026

Reference: USEPA Method 245.6.

1. Scope and Application

This method is designed for the digestion and analysis of total mercury in tissue and biota samples as referenced in EPA Method 245.6. The Reporting Detection Limit (RDL) for this procedure is <0.01 mg/kg based on an initial sample weight of 0.3 grams.

2. Summary of Method

Approximately 0.3 grams of sample is accurately measured for analysis and digested at 95°C with a mixture of sulphuric acid, nitric acid and an excess of potassium permanganate, along with potassium persulphate. Following digestion, excess potassium permanganate is destroyed with hydroxylamine hydrochloride and samples are analyzed for mercury using an automated CVAAS Analyzer. During analysis, the digested samples are mixed with stannous chloride which reduces the mercury to its atomic state. The mercury is sparged from solution using nitrogen gas and the vapour is swept into the absorption cell. The instrument signal (absorbance) at 253.7 nm is proportional to the concentration of mercury in the sample.

3. Quality Assurance

Certified reference materials, method blanks, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of 1 for every 20 samples.

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METHOD SUMMARY

Trace Metals in Biota Samples

SOP #: ATL SOP-00030

Reference: Based on EPA Method #200.3, Sample Preparation Procedure for Spectrochemical Determination of Total Recoverable Elements in Biological Tissues.

1. Scope and Application

This method is applicable to the digestion and analysis of tissue samples for the determination of trace metals. Lower Reporting Detection Limits (RDLs) or additional parameters may be available upon request.

Analyte	RDL (mg/kg)	Analyte	RDL (mg/kg)
Aluminum	2.5	Manganese	0.5
Antimony	0.5	Molybdenum	0.5
Arsenic	0.5	Nickel	0.5
Barium	1.5	Potassium	30
Beryllium	0.5	Rubidium	0.5
Bismuth	0.5	Selenium	0.5
Boron	1.5	Silver	0.12
Cadmium	0.05	Sodium	30
Calcium	30	Strontium	1.5
Chromium	0.5	Thallium	0.02
Cobalt	0.2	Tin	0.5
Copper	0.5	Titanium	0.5
Iron	15	Uranium	0.02
Lead	0.18	Vanadium	0.5
Lithium	0.5	Zinc	1.5
Magnesium	30		

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2. Summary of Method

"Available" elements are dissolved by digestion with a strong nitric acid solution. The multi-elemental determination of trace metals by ICP-MS is accomplished by measuring the ions produced when the dissolved sample is introduced into radio frequency inductively coupled plasma. These ions are sorted according to their mass-to-charge ratios and quantified with a discrete dynode electron multiplier detector.

3. Quality Assurance

Certified reference materials, method blanks, blank spikes, matrix spikes, and matrix duplicates are analyzed at a minimum frequency of one for every 20 samples.

METHOD SUMMARY

Polycyclic Aromatic Hydrocarbons in Fish Tissues/Biota

SOP #: ATL SOP-000104

Reference: USEPA Method 8270D - Semivolatile Organic Compounds By Gas Chromatography/Mass Spectrometry; Multiresidue Methods for the Determination of Chlorinated Pesticides and Polychlorinated Biphenyls (PCBs) in wildlife Tissues by Gas Chromatography/Mass Spectrometry; Canadian Wildlife Service Technical Report #335; San Francisco Estuary Institute Regional Monitoring; USEPA Method 3630C - Silica Gel Clean Up

1. Scope and Application

This method is applicable to the determination of polycyclic aromatic hydrocarbons (PAHs) in tissues and biota with LOQs of 0.05 mg/kg on a wet weight basis. The following compounds are routinely determined:

Analyte	Analyte	Analyte
Naphthalene	Anthracene	Benzo[*j*]fluoranthene
1-Methylnaphthalene	Fluoranthene	Benzo[*a*]pyrene
2-Methylnaphthalene	Pyrene	Perylene
Acenaphthylene	Benz[*a*]anthracene	Indeno[*1,2,3-cd*]pyrene
Acenaphthene	Chrysene	Dibenz[*a,h*]anthracene
Fluorene	Benzo[*b*]fluoranthene	Benzo[*ghi*]perylene
Phenanthrene	Benzo[*k*]fluoranthene	

Other PAHs can be analyzed by this method provided appropriate standards are available.

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2. Summary of Method

The tissue is homogenized in a blender and a 5 g portion is weighed out and spiked with 3 deuterated surrogate PAH compounds (these compounds represent a range of volatilities and are used to monitor the efficiency of the sample preparation steps) and extracted with hexane. An aliquot of the hexane extract is removed and interfering compounds are eliminated using a silica gel column clean-up procedure. The extract is then solvent exchanged into isooctane and analyzed by gas chromatography/mass spectrometry (GC/MS) using selected ion monitoring mode.

The GC/MS system is calibrated with PAH standards of known concentration. The areas of the individual peaks are integrated and automatic data analysis routines within the instrument software are used to store the mass spectra of the various peaks and to prepare the calibration curves. Calibration accuracy is verified by analyzing an independent reference standard. The day-to-day stability of the calibration is confirmed by analyzing calibration check solutions with each batch of samples. The components in the samples are identified using retention time criteria and qualifier ion ratios. After being detected, the individual peaks are integrated and quantified. Results are reported on an as received basis.

3. Quality Assurance

A method blank, spiked blanks (each individual PAH), matrix spike (each PAH spiked onto a tissue sample), and a replicate sample are analyzed with each batch of twenty samples. The spiked blank QC results are control charted and must meet specific acceptance criteria before sample results are released.

Fish Health Indicators	American Plaice
Sexual Maturity	
Sex Ratio	Ratio of male and female fish caught at both sites
Maturity Stage	Stage of maturity for both male and female fish using standard codes established for groundfish in the Newfoundland offshore area.
Physical Characteristics and Condition Indices	
Morphometrics	Commonly measurements of fish weight and size as well as organ weight.
Total body weight	Expressed in grams.
Gutted body weight	Total body weight minus the digestive tract.
Length	Length measured from tip of snout to end of caudal fin (or total length).
Liver weight	Expressed in grams.
Gonad weight	Expressed in grams.
Age	Expressed in years.
Condition indices	Condition indices relate the physical dimensions of an animal or one of its internal organs (e.g., liver, gonads, spleen) with body weight. They are used as a simple method for monitoring major changes in fish health.
Fulton's condition index (FCI)	FCI is defined as the ratio of body weight to length^3 X 100. It expresses the condition of a fish, such as the degree of well-being, relative robustness, plumpness or fatness in numerical terms. Changes in condition index can indicate a change in nutritional or energy status, metabolism or gonadal status. Condition factor could also vary in either direction outside the normal range in response to chemical exposure.
Hepatosomatic index (HSI)	HSI is defined as the ratio of liver weight to body weight X 100. It provides an indication of the status of energy reserves in an animal. In a poor environment, fish usually have a smaller liver (with less energy reserved in the liver). HSI could also vary in either direction outside the normal range in response to chemical exposure.
Gonadosomatic index (GSI)	GSI is defined as the ratio of gonad weight to body weight X 100. It provides information on gonadal health and reproductive status. GSI is affected by season (which controls reproductive cycling) and age. Pollutants may also cause changes in GSI.
Mixed Function Oxygenase (MFO) Activity	
MFO Activity	The MFO system refers to a family of enzymes that transforms the structure of organic chemicals and performs a critical role in detoxification and other physiological processes. The activity of this enzyme system commonly increases in animals in the presence of certain classes of organic pollutants. Measurements of the activity have been used as an indicator of exposure and toxicity to certain class of pollutants.
Gross Pathology	
Gross Pathology	Gross pathology refers to the observation and quantification of the presence of visible diseases, lesions and other abnormalities on the skin or fins and on internal organs. Specific organs analyzed include skin, fins, spleen, hindgut, kidney, liver, eyes, gills, and parasite load.
Health Assessment Index	The health assessment index assigns scores to individual organ pathologies and gives individual fish a score based on incidence and severity of pathologies.
Haematology	
Cell type frequencies	The proportion of lymphocytes, neutrophils, and thrombocytes is compared.
Lymphocytes	Small white blood cells with a large nucleus and a small amount of cytoplasm – cells important in immunity.

Fish Health Indicators	American Plaice
Neutrophils	Large white blood cells with a nucleus that is rod or kidney shaped and on occasion is bi-lobed - cells important in immunity.
Thrombocytes	Oval or spindle cells with projection(s) of cytoplasm in one or both ends of the cell - cells important in blood clotting.
Histopathology	
Histopathology	Histopathology involves the analysis of tissues for the presence of cellular damage (i.e., lesions and tumours) which can indicate chronic, or long-term, exposure to pollutants.
Liver Histopathology	
Nuclear pleomorphism	Variation in the diameter of cell nuclei.
Nonspecific necrosis	Tissue necrosis not specific to another pathology.
Bile duct hyperplasia	Proliferation of cells in the bile duct
Megalocytic hepatosis	Lesion characterised by an increase in nuclear and cellular diameters of liver cells and various cytoplasmic degenerative changes.
Eosinophilic foci	Foci of cellular alteration staining with eosin.
Basophilic foci	Foci of cellular alterations having an affinity for basic dyes.
Clear cell foci	Foci of cellular alteration which are non-staining.
Hepatocellular Carcinoma	Any cancer that arises from epithelial cells.
Benign tumours	Tumours that are not malignant
Cholangioma	Tumour of the bile duct.
Cholangiofibrosis	Increase in fibrous connective tissue around bile ducts.
Increase in mitotic activity	Increase in cell division.
Macrophage aggregation	Aggregation(s) of phagocytic white blood cells in tissues.
Hepatocellular vacuolation	Alteration in the number or in the distribution of vacuoles in cells.
Hydropic vacuolation	Formation of many non-staining cytoplasmic vacuoles in cells.
Fibrillar inclusions	Inclusions appearing as bundles at various orientations within cells.
Gill Histopathology	
Distal hyperplasia	Thickening of the epithelium around the two sides of the secondary lamellae due to an increase in the number of epithelial cells.
Epithelial lifting	Separation of the epithelial layer from the basement membrane.
Telangiectasis	Dilatation of blood vessel at the tip of the secondary lamellae.
Basal hyperplasia	Thickening of the epithelium near the base of secondary lamellae due to an increase in the number of epithelial cells.
Tip hyperplasia	Thickening of the epithelium around the secondary lamellar tip due to an increase in the number of epithelial cells.
Fusion	Fusion of two or more adjacent secondary lamellae.
Oedema condition	Swelling within cells.