CHAPTER 9

TIER III: BIOLOGICAL TESTING

9.1 OVERVIEW

Biological effects tests may be necessary if Tier I or Tier II evaluations indicate that the dredged material contains contaminant concentrations which may be harmful to aquatic organisms. Tier III biological testing of dredged material will be required when chemical testing results exceed guideline values. A standard suite of bioassays is used to make a determination regarding the suitability of the dredged material for aquatic disposal. Tests involving whole sediment determine the potential effects for bottom-dwelling organisms. Tests using suspension/elutriates of dredged material are used to assess the potential effects on water column organisms. A bioaccumulation test is required when certain chemicals of concern are detected at concentrations which may pose a potential risk to human health or ecological health in the aquatic environment (Chapter 8).

Prior to the 1980s, the assessment of water and sediment quality was often limited to physical and chemical characterizations. However, quantifying chemical concentrations alone is not always adequate to assess potential adverse environmental effects, interactions among chemicals, or bioavailability of chemicals to aquatic organisms. Because the relationship between total chemical concentrations and biological availability is poorly understood, when regulatory limits are exceeded, controlled laboratory bioassay and bioaccumulation tests are performed to assess environmental effects.

The approach most often adopted is to expose representative aquatic species for relatively short periods of time: up to 10 days for acute toxicity, up to 20 days to assess potential chronic/sublethal effects, and 28 days to assess bioaccumulation potential. These tests provide information about different possible biological effects. In addition, testing multiple species reduces uncertainty about the results and limits errors in interpretation.

This chapter includes information on which biological test species should be used, on the quality control requirements for each test, and on the interpretive criteria used for decision-making. References are provided for more detailed information on test protocols and test interpretation.

9.2 SEDIMENT SOLID PHASE BIOLOGICAL TESTS

Biological testing can be conducted to measure effects on organisms exposed to the water column or to whole sediment. The biological testing suite discussed in this section addresses solid phase toxicity testing using whole sediment. Both marine and freshwater species are specified. Several biological tests are under development/review, and may be added in the future. Biological test species are selected based on the salinity conditions at the disposal site for the dredged material. For projects in the Lower Columbia River Management Area, the use of the Ocean Disposal sites will require marine bioassays (if bioassay testing is required).

9.2.1 Marine Bioassays

10-day amphipod acute mortality test
- *Rhepoxynius abronius*
- *Ampelisca abdita*[1]
- *Eohaustorius estuarius*[2]

Chronic Tests
- *Neanthes arenaceodentata* (Los Angeles karyotype) 20-day growth test

Sediment larval test
- Echinoderm
  - *-Dendraster excentricus*[3]
  - *-Strongylocentrotus purpuratus*
  - *-Strongylocentrotus droebachiensis*
- Bivalve
  - *-Crassostrea gigas*
  - *-Mytilus provincialis*

The protocols to be used to run the recommended marine bioassays are described by the Puget Sound Estuary Program (PSEP), and can be found in *Recommended Guidelines for Conducting Laboratory Bioassays on Puget Sound Sediments* (PSEP,

[1]may be substituted if test sediment contains greater than 60% fines.

[2]may be considered for substitution if test sediment is greater than 60% fines and salinity is less than 25 ppt.

[3]recommended species.

1995). Project applicants should contact the DMMO/DMMT or the Puget Sound Water Quality Action Team for recent protocol updates. The PSEP protocols are consistent with national guidance on bioassay testing.

Amphipod Species Substitution**.** *Rhepoxynius abronius* has been shown to be responsive to high percent fines in sediments, particularly high clay content sediments, and has been shown to exhibit mortalities greater than 20 percent in clean, reference area sediments with this grain-size(DeWitt et al, 1988; Fox, 1993). Applicants may wish to consider substituting *Ampelisca abdita* for *Rhepoxynius abronius* when fines exceed 60 percent. *Ampelisca* is relatively grain-size-insensitive to concentrations of fines greater than 60 percent. Any proposed species substitutions must be submitted to the DMMO/DMMT for approval by the RMT prior to use, and the substitutions must be documented in the sampling and analysis plan for the proposed dredging project.

9.2.2 Freshwater Bioassays. The following freshwater bioassays will be required when the proposed disposal location for dredged material is in a low salinity (generally 5 parts per thousand or below) environment.

Amphipod - *Hyalella azteca* 10 day Survival Test

Midge - *Chironomus tentans* 10 day Survival and Growth Test

Standard protocols exist for each of these tests, established both by ASTM and EPA (ASTM 1995, EPA 1994). Either protocol may be used for the freshwater bioassays. The protocols specify negative control, positive control, and test performance criteria. Adherence to these performance standards aids in interpreting bioassay responses by limiting effects from factors other than sediment toxicity. Other biological tests to measure chronic effects in freshwater are still under development. One may be added to this test suite in the future.

9.2.3 Bioassay Testing Performance Standards. This section contains the specific quality assurance/quality control requirements for solid phase biological testing. The parameters covered include:

- Negative Control and Reference Samples
- Quality Control Limits for the Negative Control Treatment
- Quality Control Limits for the Reference Treatment
- Reference Toxicant
- Water Quality Monitoring

General procedures are given first, followed by specific performance standards for each bioassay. These standards aid in interpreting the bioassay responses, since they control for environmental effects which may produce effects not associated with toxicity.

Negative Controls. Negative control sediments are used in bioassays to check laboratory performance. Negative control sediments are clean sediments in which the test organism normally lives and which are expected to produce low mortality. Control reliability must be demonstrated.

The sediment larval test utilizes a negative seawater control rather than a control sediment. The seawater control will be collected from a location approved by the DMMO/DMMT.

Reference Sediment. Agency regulations prescribe the use of bioassay reference sediments for test comparison and interpretations which closely match the grain size characteristics of the dredged materials test sediments. The reference sediment provides a point of comparison for evaluating the potential effects of the dredged material. If chemical concentrations in the reference area are not well-documented, a complete characterization may be required.

All bioassays have performance standards for reference sediments. Failure to meet these standards may result in the requirement to retest.

All reference sediments will be analyzed for total solids, total volatile solids, total organic carbon, ammonia, sulfides and grain-size.

Replication. Five laboratory replicates of test sediments, reference sediments and negative controls will be run for each bioassay.

Positive Controls. A positive control will be run for each bioassay. Positive controls are chemicals known to be toxic to the test organism and which provide an indication of the sensitivity of the particular organisms used in a bioassay.

Water Quality Monitoring. Water quality monitoring of the overlying water will be conducted for the bioassays. This consists of daily measurements of salinity, temperature, pH and dissolved oxygen for the amphipod and sediment larval tests. These measurements will be made every three days for the *Neanthes* bioassay. Ammonia and sulfides will be determined at test initiation and termination for all tests. Monitoring will be conducted for all test and reference sediments and negative controls (including seawater controls). Parameter measurements must be within the limits specified for each

bioassay. Measurements for each treatment will be made on a separate chemistry beaker set up to be identical to the other replicates within the treatment group, including the addition of test organisms.

Bioassay-specific Procedures - Marine

Amphipod Bioassay. This test involves exposing amphipods to test sediment for ten (10) days and counting the surviving animals at the end of the exposure period. Daily emergence data and the number of amphipods failing to rebury at the end of the test will be recorded as well. The control sediment has a performance standard of 10 percent mortality. The reference sediment has a performance standard of 20 percent mortality greater than control. Test species selection is discussed in Section 9.2.1

Sediment Larval Bioassay. This test monitors larval development of a suitable echinoderm species in the presence of test sediment. The test is run until the appropriate stage of development is achieved in a sacrificial seawater control. At the end of the test, larvae from each test sediment exposure are examined to quantify abnormality and mortality.

The seawater control has a performance standard of 30 percent combined mortality and abnormality. The reference sediment has a performance standard of 35 percent combined mortality and abnormality greater than the seawater control performance.

Initial counts will be made for a minimum of five 10-ml aliquots. Final counts for seawater control, reference sediment and test sediment will be made on 10-ml aliquots.

The sediment larval bioassay has a variable duration (not necessarily 48 hours) which is determined by the developmental stage of organisms in a sacrificial seawater control.

Ammonia and sulfides toxicity may interfere with test results for this bioassay. Aeration will be conducted throughout the test to minimize these effects if required.

***Neanthes* Growth Test.** This test utilizes the polychaete *Neanthes arenaceodentata*, in a 20-day growth test. The growth rate of organisms exposed to test sediments is compared to the average individual growth rate of organisms exposed to a reference sediment. The control sediment has a performance standard of 10 percent mortality. The reference sediment has a performance standard of 80 percent of the control average individual growth rate and 20 percent mortality.

Bioassay-specific Procedures - Freshwater

Amphipod Bioassay. This bioassay measures the survival of amphipods after a 10-day exposure to the test sediment. The control has a performance standard of 20 percent absolute mean mortality. The reference sediment performance standard is 30 percent absolute mean mortality.

Midge Bioassay. This test measures the survival and growth of the midge *Chironomus tentans* after a 10-day exposure to the test sediment. The control has a performance standard of 30 percent absolute mean mortality and a growth performance standard of 0.6 mg minimum mean weight per organism (per ASTM). The reference performance standard is 35 percent absolute mean mortality.

9.2.4 Bioassay Interpretive Criteria. The response of bioassay organisms exposed to the tested dredged material representing each management unit will be compared to the response of these organisms in both control and reference treatments. This will determine whether the material is suitable for unconfined aquatic disposal.

Biological test interpretation relies on two levels of observed response in the test organisms. These are known as one-hit or two-hit failures. The bioassay-specific guidelines for each of these response categories is listed below. In general, a one-hit failure is a marked response in any one biological test. A two-hit failure is a lower intensity of response. It must be found in two or more biological tests in order for the sediment to be found unsuitable for aquatic disposal.

(1) One-Hit Failure. When **any one** biological test shows a test sediment response relative to the negative control and reference sediment which exceeds the bioassay-specific response guidelines, and which is "statistically different" from the reference, the dredged material management unit is judged to be unsuitable for aquatic disposal. The acceptable methods for determining statistical significance are in Appendix 9-A.

(2) Two-Hit Failure. When **any two** biological tests show test sediment responses, which are less than the bioassay specific guidelines noted above for a single-hit failure, but show a lower level effect and are significantly different statistically from the reference sediment, the dredged material management unit is judged to be unsuitable for aquatic disposal.

This interpretation of solid phase biological test results will be used for both the CWA Section 404(b)(1) evaluation/Section 401 water quality certification process, and for the MPRSA Section 103 evaluation process. The application of these interpretive guidelines to a set of sample test results is described in Appendix 9-B.

The determination of a "statistically different " response involves two conditions: first, the response in the tested dredged material management unit must be greater than 20 percent different from the control response; and second, that a statistical comparison between mean test and mean reference responses must show a significant difference. The appropriate method for making the latter determination is discussed in Appendix 9-A. This appendix also contains a description of the Biostat bioassay software developed by the Corps of Engineers. This software contains the appropriate statistical tests to determine sediment suitability.

Marine Bioassays

Amphipod Bioassay. For the amphipod bioassay, mean test mortality greater than 20 percent absolute over the mean negative control response, and greater than 30 percent absolute over the mean reference sediment response, and statistically different from the reference (alpha = 0.05), is considered a "one-hit".

Juvenile Infaunal Growth Test. Juvenile *Neanthes* growth test results that show a mean test individual growth rate less than 80 percent of the mean negative control growth rate, and less than 50 percent (relative) of the mean reference sediment growth rate, and statistically different from the reference (alpha = 0.05), is considered a "one-hit".

Sediment Larval Bioassay. For the sediment larval bioassay, test and reference sediment responses are normalized to the negative seawater control response. This normalization is performed by dividing the number of normal larvae from the test or reference treatment at the end of the exposure period by the number of normal larvae in the seawater control at the end of the exposure period, and multiplying by 100 to convert to percent. The normalized combined mortality and abnormality (NCMA) is then 100 minus this number. If the mean NCMA for a test sediment is greater than 20 percent, and is 30 percent absolute over the mean reference sediment NCMA, and statistically different from the reference (alpha = 0.10), it is considered a "hit".

Freshwater Bioassays

Amphipod Bioassay. For the amphipod bioassay, mean test mortality greater than 15 percent over the mean reference response, and statistically different from the reference (alpha = 0.05), is considered a "hit".

Midge Bioassay. For the midge mortality test, a mean mortality in the test sediment of 20 percent over reference and statistically different from reference (alpha = 0.05) is a hit. For the growth test, a mean reduction in biomass greater than 40% and statistical significance is considered a "hit". If either or both endpoints fail the guideline, the test is considered a "hit".

9.3 WATER COLUMN BIOASSAY TESTING

The Tier III evaluation of dredged material will include an evaluation of potential water column effects when warranted. Water column testing for biological effects is not routinely required for regulated or federal dredging projects evaluated under CWA Section 404. The test is required under MPRSA Section 103 for ocean disposal when biological testing is required. This test will need to be conducted only when the water quality certification agency (Washington Department of Ecology or Oregon Department of Environmental Quality for Section 404/401 permits or the Environmental Protection Agency for Section 103 ocean disposal permits) requires an assessment of potential water column toxicity effects relative to a particular chemical of concern.

In the event that water column testing is required, one of the following tests will be conducted. The appropriate assessment is described in the Ocean Testing Manual (EPA/USACE 1991) and the Inland Testing Manual (USACE/EPA 1998). The interpretation guidelines specified in either manual will be used, depending on whether the ultimate disposal environment proposed is in the Section 103 (ocean) or in 404 (fresh water, estuarine, or near coastal) waters. Protocols for the water column test should follow the test specification requirements described in the Inland Testing Manual (Appendix E). The following species may be used for the water column bioassay test:

Marine

T Echinoderm
- *Dendraster excentricus*
- *Strongylocentrotus purpuratus*
- *Strongylocentrotus droebachiensis*

T Bivalve

- *Crassostrea gigas*
- *Mytilus provincialis*

Freshwater

TCrustaceans

Daphnia magna
Ceriodaphnia dubia

TFish

Pimephales promelas

9.4 BIOACCUMULATION TESTING

The Ocean Testing Manual and Inland Testing Manual provide information necessary to estimate the potential for bioaccumulation to occur. Plausible exposure scenarios, using the theoretical bioaccumulation potential (TBP) approach, were developed. The outcome of these assessments were the bioaccumulation triggers of chemicals likely to be assimilated in aquatic tissue. These reason-to-believe triggers serve as a surrogate for the TBP approach outlined in the OTM and ITM. When non-polar organic compounds (other than those on our existing list of chemicals of concern) are identified for individual projects, the TBP model will be run for those compounds.

Body burdens of chemicals are of concern for both ecological and human health reasons. A bioaccumulation test in Tier III will normally only be conducted on those dredged materials in which a reason-to-believe has been established that specific chemicals of concern may be accumulated in the tissues of target organisms. Bioaccumulation testing evaluating exposures to two species will be required when any given sediment chemical level exceeds any bioaccumulation trigger value. These values establish the reason-to-believe levels for chemicals likely to bioaccumulate. Bioaccumulation of compounds listed in Appendix 9-C should be detectable, following a 28-day exposure period, even though steady state may not have been reached. The purpose of a Tier III bioaccumulation test is not to determine steady state bioaccumulation rate (this is accomplished in Tier IV), but to assess the potential for bioaccumulation.

Following a comparison of residue levels in dredged material exposed organisms to FDA action levels, a statistical comparison is made between organisms exposed to dredged material and organisms exposed to a suitable reference material. No adverse effects are likely if the concentration in the dredged material exposed tissue is less than that in the reference exposed tissue. A higher concentration, however, does not

necessarily mean adverse effects. Additional contaminant-specific information is required to determine if adverse effects are likely.

To assist in making determinations about the likelihood for effects, USACE Waterways Experiment Station, and EPA have developed the Environmental Residue-Effects Database (ERED). The database contains over 2000 records including information on more than 200 contaminants and 100 aquatic species. ERED can be accessed at http://www.wes.army.mil/el/ered. For those compounds at statistically elevated concentrations in dredged material-exposed organisms, making determinations about the likelihood for adverse effects should be based on measurable effects listed in the ERED. Not all effects are created equal. Data in ERED may reflect a particular tissue residue, and may be species specific. Cellular/subcellular responses are an indication of organism stress, but the causal relationship between these effects and higher order effects is unknown in most cases.

The Inland Testing Manual requires two bioaccumulation tests utilizing species from two different trophic niches representing a suspension-feeding/filter-feeding and a burrowing deposit-feeding organism. A Tier III 28-day bioaccumulation test will conduct an evaluation with both an adult bivalve (*Macoma nasuta*) and an adult polychaete (*Nereis virens, Nepthys,* or *Arenicola marina*) for marine sediments. For freshwater sediments, the test will be conducted with the oligochaete *Lumbriculus variegatus* and another species to be determined at the time of testing**.** The test exposure duration will be 28 days utilizing the EPA protocol (Lee *et al* 1989), after which a chemical analysis will be conducted of the tissue residue to determine the concentration of selected chemicals of human health concern, and to assess ecological effects through a statistical comparison with a suitable reference area sediment. Protocols for tissue digestion and chemical analysis will follow the PSEP recommended procedures for metals and organic chemicals.

Human Health. The bioaccumulation test results are compared to guideline values to determine exceedance of allowable tissue residue concentrations. If the 28-day bioaccumulation test results in tissue levels greater than the FDA action levels, (see Table 3, Appendix 9-B) or agency guidelines in effect at the time, the sediment will be considered unsuitable for aquatic disposal. Chemicals of concern without or below FDA action levels will be evaluated by the RMT using best professional judgment and risk assessment approaches.

Ecological Effects. The results of a Tier III 28-day bioaccumulation test will be compared directly with reference results for statistical significance. If the results of a statistical comparison show that the tissue concentration of the chemical(s) of concern

tested in sediments is statistically different (t-test, alpha level of 0.05) from the reference sediment, the dredged material will generally be considered unsuitable for unconfined aquatic disposal.

If results of the bioaccumulation test in Tier III are found to be equivocal, further testing may be required in Tier IV before a regulatory decision can be made on the suitability of the dredged material for unconfined open-water disposal. An exposure period of 28 days may be insufficient for the test species selected to achieve a steady state tissue concentration in a normal Tier III bioaccumulation test.

Bioaccumulation testing for the assessment of dredged material is currently under Corps/EPA review. Additional guidance will be added to this manual as it becomes available.

9.5 REFERENCE SEDIMENT COLLECTION SITES

Bioassays must be run with a reference sediment which is well-matched to the test sediments for grain-size, and for other sediment conventionals such as total organic carbon and must match the disposal environment. The sampling protocol used for the collection of a reference sediment can affect its performance during biological testing. The following guidelines should be followed when collecting reference sediments:

- Use experienced personnel
- Follow protocols
- Sample from biologically active zone
- Avoid anoxic sediment below the Redox Potential Discontinuity (RPD) horizon
- Use wet-sieving method

The wet-sieving protocol is used in the location of an appropriate reference station. Wet-sieving is imperative in finding a good grain size match with the test sediment. Wet-sieving is accomplished using a 63-micron (#230) sieve and a graduated cylinder; 100 ml of sediment is placed in the sieve and washed thoroughly until the water runs clear. The volume of sand and gravel remaining in the sieve is then washed into the graduated cylinder and measured. This represents the coarse fraction; the

fines content is determined by subtracting this number from 100. Wet-sieving results will not perfectly match the dry-weight-normalized grain size results from the laboratory analysis, but should be relatively close.

The Corps of Engineers and EPA have identified locations suitable as reference stations. Reference site selection will be made on a case-by-case basis with information and guidance provided by the Corps and EPA. Reference site grain-size should match, as closely as possible, that of the test sediment and the disposal environment. In the absence of a match, the agencies will select a coarser grained sediment for use. This is likely to yield better test performance, and to be environmentally conservative. Reference site selection and reference sample collection must be coordinated with the DMMO/DMMT.