

**Table 7. The predictive reliability of amphipod mortality and echinoderm larvae abnormality AETs.** 1994 AETs (shaded) are compared to original 1988 amphipod and 1986 oyster AETs. "Sensitivity," "Efficiency," "Overall Reliability," "Independent AETs," and "Pooled" are defined in the Background and Glossary sections of this report. Measures of reliability are given as percentages and fractions. Historical reliability values are from "1988 Update and Evaluation of Puget Sound AET" (4).

AET Group	Database for Comparison	Total Samples	Sensitivity	Efficiency	Overall Reliability
1988 HAETs	1988		NA	NA	NA
1988 HAETs	1994		NA	NA	NA
1988 HAETs	1994 Pooled		NA	NA	NA
1994 HAETs	1994		NA	NA	NA
1994 HAETs	1994 Pooled		NA	NA	NA
Original PSDDA MLs	1988		NA	NA	NA
1994 PSDDA MLs	1994		NA	NA	NA
1994 PSDDA MLs	1994 Pooled		24% (75/309)	77% (75/98)	NA
Proposed New PSDDA MLs	1994		NA	NA	NA
1988 LAETs	1988		NA	NA	NA
1988 LAETs	1994		NA	NA	NA
1988 LAETs	1994 Pooled		NA	NA	NA
1994 LAETs	1994		NA	NA	NA
1994 LAETs	1994 Pooled		NA	NA	NA
Original PSDDA SLs	1988		NA	NA	NA
1994 PSDDA SLs	1994		NA	NA	NA
1994 PSDDA SLs	1994 Pooled		84% (260/309)	51% (260/514)	NA
1994 HAET/10	1994		NA	NA	NA
Proposed New PSDDA SL	1994		NA	NA	NA

**Possible Additional PSDDA Reliability Analyses:**

**Table 7. The predictive reliability of amphipod mortality and echinoderm larvae abnormality AETs.** 1994 AETs (shaded) are compared to original 1988 amphipod and 1986 oyster AETs. "Sensitivity," "Efficiency," "Overall Reliability," "Independent AETs," and "Pooled" are defined in the Background and Glossary sections of this report. Measures of reliability are given as percentages and fractions. Historical reliability values are from "1988 Update and Evaluation of Puget Sound AET" (4).

AET Group	Database for Comparison	Total Samples	Sensitivity	Efficiency	Overall Reliability
1988 2AETs	1988		NA	NA	NA
1988 2AETs	1994		NA	NA	NA
1988 2AETs	1994 Pooled		NA	NA	NA
1994 2AETs	1994		NA	NA	NA
1994 2AETs	1994 Pooled		NA	NA	NA
1991 CSLs/MCULs	1994		NA	NA	NA
1991 CSLs/MCULs	1994 Pooled		NA	NA	NA
Proposed New CSL/MCUL	1994		NA	NA	NA
1988 LAETs	1988		NA	NA	NA
1988 LAETs	1994		NA	NA	NA
1988 LAETs	1994 Pooled		NA	NA	NA
1994 LAETs	1994		NA	NA	NA
1994 LAETs	1994 Pooled		NA	NA	NA
1991 SQSs	1994		NA	NA	NA
1991 SQSs	1994 Pooled		NA	NA	NA
Proposed New SQS			NA	NA	NA

**Possible Additional SMS Reliability Analyses:**

**Table 8.** Number and fraction of incorrectly predicted "Hit" samples having elevated levels of bulk ammonia (> 20 ppm), percent fines (>80% silt+clay), bulk sulfides (> 50 ppm). NA = data not available.

<b>AET Type, Normalization</b>	<b>High Ammonia</b>	<b>High Fines</b>	<b>High Sulfides</b>	<b>≥ 2 High Conventionals</b>
Amphipod, dry weight	21/26	40/88	34/57	31/104
Amphipod, TOC	NA	NA	NA	NA
Echinoderm, dry weight	35/41	9/29	25/32	24/47
Echinoderm, TOC	NA	NA	NA	NA

However, neither of those actions was taken. There was no clear predominance of "Hit" samples among those having greater than 80% fines. And, while the PSDDA agencies have found a significant correlation between percent fines and amphipod mortality (11), the correlation appeared to be of limited utility for excluding high percent fines "Hit" samples from 1994 AET calculations. So, while there was perhaps a potential case for conventional parameters influencing determinations of adverse effects, thereby lowering the sensitivity of AETs, there appeared to no objective tool by which that determination could easily be improved.

### **Reliability of Pooled AETs**

As described in Methods (page 20), a pooled reliability analysis considers a sample exhibiting a significant adverse effect of any type - amphipod mortality, benthic abundance, echinoderm larval abnormality, Microtox luminosity, or oyster larval abnormality - to be a "Hit" sample, even if it showed no other adverse

effects. There were 309 such "Hit" samples in the 1994 AET database<sup>17</sup>. Using those data, PSDDA agencies calculated some pooled reliability values for the 1994 suite of five Puget Sound AETs. Those limited results appear in Table 7 and are described below.

When all biological effects samples were pooled, the most sensitive 1994 dry weight-normalized AET type was Microtox<sup>®</sup> luminosity. 1986 dry weight-normalized Microtox<sup>®</sup> AET values correctly predicted 92% of all samples showing at least one type of "Hit." The other four 1994 dry weight-normalized Puget Sound AETs, ranked from most to least sensitive, were: 1986 oyster larval abnormality (76%), 1994 echinoderm larval abnormality (67%), 1988 benthic abundance (51%), and 1994 amphipod mortality (31%). The pooled efficiency of 1994 dry weight-normalized AETs, in descending order, were 88% for amphipod mortality, 59% for echinoderm larval abnormality, 51% for oyster larval abnormality, 49% for benthic abundance, and 45% for Microtox<sup>®</sup> luminosity. The overall reliability of 1994 dry weight-normalized AETs was highest for amphipod mortality (71%), followed by echinoderm abnormality (68%), oyster abnormality (61%), benthic abundance (59%) and Microtox<sup>®</sup> luminosity (53%).

Results of pooled reliability analysis for 1994 TOC-normalized AETs revealed that the amphipod mortality AETs were the least sensitive (28%) but the most efficient (65%) and overall reliable (65%). The Microtox<sup>®</sup> AETs had the highest pooled sensitivity (79%) but were the least efficient (44%) and least reliable overall (52%). The other AET groups were intermediate in all the measures of reliability when adverse effects were pooled for synoptic samples.

PSDDA agencies also calculated the pooled reliability for the 1994 LAET, 2AET and HAET values. Results of those calculations are still being tabulated and verified. Additional reliability calculations are suggested in Table 7 and in Recommendations for Future Work. Other reliability analyses of 1994 AETs may be recommended by technical and regulatory experts reviewing this document.

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<sup>17</sup> This number is less than the total given in Table 2 (414) because many samples exhibit more than one significant adverse biological effect.

## CONCLUSIONS AND DISCUSSION

### The 1994 AET Database

The size and nature of the 1994 Puget Sound sediment quality values database changed significantly between 1988 and 1994. Newly-obtained synoptic samples nearly tripled the SEDQUAL total from 334 to 924. The total number of determinations of significant adverse effects of all types which were used to calculate AETs rose from 594 in 1988 to 1186 in 1994.

Approximately 70% of all the 1988 synoptic surveys (8/11) and samples (235/334) were conducted in or collected from primarily urban areas, with about 80% of the biological effects samples (469/594) collected from urban areas. "Hit" samples constituted nearly one-half of those (227/469), or 38% of the total (227/594) in the 1988 AET database.

By comparison, only about 55% of the newly-obtained surveys (22/41) and synoptic samples (332/590) were urban. Less than half of the urban stations (154/332) and only 26% of the total number of biological effects samples (154/590) were determined to be "Hit" samples. Two urban surveys, PIERD\_91 and SITCUMRI, accounted for 44% (68/154) of all new "Hit" samples.

Just how well did the 1988 AET database represent Puget Sound in its entirety? How representative is the 1994 AET database? Those questions do not have simple answers, but the 1994 sediment quality values database is clearly less urban in character than the 1988 sediment quality values database.

### Methods

PSDDA agencies' stringent QA review of chemical and bioassay data resulted in exclusion of many synoptic sample data. Over one-half of all newly-obtained amphipod mortality samples (452/833) were not used in 1994 AET calculations. Nearly all new bivalve larval bioassay samples failed to meet QA guidelines or lacked a suitable reference area sample to which test sample responses could be compared. That was also true for one-third of all echinoderm samples (112/317).

Most of the samples excluded from 1994 AET calculations lacked at least one adequate reference sample. In many cases that was intentional; the purpose of certain types of surveys did not include comparison of test to reference samples. An example of such a survey was the Puget Sound Reference Areas Study (PSREF90). Others used local "background" sites which did not adhere to the PSEP definition of reference area. Examples of those included many

Thus, it appears that the expansion of the Puget Sound AET database may depend on the careful collection and adequate performance of reference area samples. The PSDDA agencies believe that collection and performance of future reference area samples is improving. Recent surveys often collect more than one reference sample, in the event one does not meet performance standards. Concern about possible future sediment cleanup liability, and so the need to correctly characterize the biological sediment quality in a specific area, may also increase the fraction of surveys which carefully collect at least one reference sample.

While most of the synoptic sample data not used in 1994 AET calculations were excluded because they lacked a suitable reference sample, relatively few were casualties of new reference sample performance standards (see Table 1 and Figures A-1, A-2, and A-3). Similarly, few samples were excluded for being statistically inconclusive (Table B-6). For example, subjecting highly variable echinoderm larval samples to a power analysis had minimal effect on the final list of samples used to calculate 1994 AETs. Of the total inventory of synoptic echinoderm samples, only 11 samples (5%) were excluded.

Exclusion of chemically anomalous samples had a much greater effect on 1994 resulting AET values. Although defined as a "No Hit" sample that was three times higher concentration than the next highest "No Hit" sample, the average anomalous sample was more than five times higher than the next highest "No Hit" sample (Table B-7). If those anomalous samples had not been excluded, individual AET values would have been higher and generally much less sensitive. Similarly, TOC-normalized AET values would have been higher and less sensitive had the chemically anomalous TOC-normalized data been retained.

### **1994 Puget Sound AET Values**

The synoptic data added to SEDQUAL during 1992 and 1993 were used to calculate new amphipod mortality AETs. A new, fifth type of AET was also calculated: echinoderm larval abnormality AETs.

The PSDDA agencies obtained several general types of 1994 AET results. First, many of the new AET values were unqualified, confirmed by at least one "Hit" sample having a higher chemical concentration. Some of those were newly-confirmed AETs, having only been estimated values in 1988. Others were altogether new, with no 1988 counterparts. Second, many new AETs could only be estimated, due in part to the nature of the 1994 database. With relatively few of the added synoptic samples collected from more contaminated areas, there were relatively fewer "Hit" samples to confirm new AET values. Finally, some AET values which were confirmed in 1988 could only be estimated in 1994. This occurred when at least one new "No Hit" sample having a higher chemical

concentration was added to the database and there was no confirming "Hit" sample with a higher concentration.

The majority of all new 1994 amphipod and echinoderm AETs which could be confirmed were from the chemical groups most frequently required in sampling and analysis plans, and most frequently detected: trace metals and PAHs.

### Amphipod Mortality

PSDDA agencies expected that merely including more "No Hit" samples in amphipod mortality AET calculations, by virtue of the definition of AETs, would drive existing values higher. Indeed, AETs increased for six trace metals, seven individual PAHs and three substituted phenols. The largest increases were for arsenic (from 93 ppm to 450 ppm), zinc (to 3,800 ppm), dibenzo(a,h)anthracene (to 1,900 ppm) and phenanthrene (to 21,000 ppb). Estimated AET values for chromium and bis[2-ethylhexyl]phthalate increased to >1100 ppm and >8300 ppb, respectively. In addition, the amphipod AETs for ethylbenzene, total xylene and p,p'-DDT were confirmed in 1994.

1994 TOC-normalized amphipod AETs also tended to be the same or greater than 1988 values. Some of the AETs for trace metals, however, decreased. That apparently resulted when a few 1988 AET "No Hit" samples, after TOC-normalization and combination with 1994 samples, were excluded for being chemically anomalous.

As stated in the Results section, one amphipod AET which showed a particularly large decrease in 1994 was for benzyl alcohol. The dry weight-normalized value decreased from 870 ppb to 73 ppb, while the TOC-normalized values dropped from 73 ppb to just 5 ppb. The reason appeared to be that the 1988 value was set by a chemically anomalous sample (CBMSQS, HY-50) which should have been excluded from previous calculations, but was not.

### Echinoderm

The PSDDA agencies could not anticipate the magnitude or reliability of a new AET type, one based on echinoderm larval abnormality. They found that the 1994 echinoderm AET values were generally lower than 1986 oyster AETs. Of the dry weight-normalized echinoderm AET values which could be confirmed, 27 were lower and seven were higher than the corresponding oyster values. Overall, TOC-normalized echinoderm AETs were also lower than oyster values, especially if estimated values were considered.

Lower echinoderm AETs might indicate a fundamentally lower tolerance of the bioassay test organisms, primarily *Dendraster excentricus*, toward chemical contaminants in Puget Sound sediments. However, without comparable echinoderm and oyster larval abnormality sample numbers, a broader geographic representation for both, and increased side-by-side testing, that conclusion would be premature.

#### **New HAETs, 2AETs, LAETs: Potential Guidelines and Standards**

Many of the 1994 amphipod and echinoderm AETs which a) increased and were confirmed, b) remained the same magnitude but were newly confirmed, or c) were for chemicals previously lacking AET values, like dieldrin, could potentially form the basis for new guidelines or standards. 1994 AET values representing new highest AET (HAET), second lowest AET (2AET) or lowest AET (LAET), could be of particular interest to regulatory programs and the regulated community, especially if there was no appreciable loss of reliability associated with those values.

For that reason, PSDDA agencies examined the new suite of five Puget Sound AETs. That was done by combining 1994 amphipod mortality and echinoderm larval abnormality AETs with the 1988 benthic abundance, 1986 Microtox luminosity and 1986 oyster larval abnormality AETs. The resulting values are detailed in Tables 9 and 10, and summarized in Table 11.

There were 17 new dry weight-normalized HAETs, all but one set by the 1994 amphipod AETs. The only new HAET value set by a 1994 echinoderm abnormality AET was for silver. HAETs increased for five trace metals (cadmium - 14 ppm, lead - 1,200 ppm, mercury - 2.3 ppm, silver - 8.4 ppm and zinc - 3,800 ppm), six PAH compounds (LPAH - 29,000 ppb, phenanthrene - 21,000 ppb, benzo[g,h,i]perylene - 3,200 ppb, chrysene - 21,000 ppb, dibenzo(a,h)anthracene - 1,900 ppb, and indeno(1,2,3-c,d)pyrene - 4,400 ppb) and six other chemicals of concern. Many of the new HAETs were for chemicals commonly found at sites proposed for dredging.

Because ML guidelines have been based on dry weight-normalized HAETs, PSDDA agencies could consider adopting new ML values for any or all of those chemicals of concern. New HAET values could also cause changes to PSDDA agencies screening level guidelines. For example, the SL for lead could be changed from 66 ppm (1/10 the current ML value of 660 ppm) to 120 ppm (1/10 the new ML of 1200 ppm).

Ten TOC-normalized HAETs also increased in 1994. However, under the current paradigm, those HAETs have no implications for regulatory programs. PSDDA program guidelines were not based on TOC-normalized AETs and SMS standards were not based on HAETs.

**Table 9. The 1994 suite of dry weight-normalized Puget Sound AETs.**

1994 amphipod mortality and echinoderm larvae abnormality AETs are combined with 1988 benthic abundance, 1986 Microtox luminosity and oyster abnormality AETs. A ">" symbol indicates a minimum value, not confirmed by a "Hit" sample with a higher concentration. New and confirmed HAETs are in boxes, 2AETs are in bold italics, and LAETs are shaded.

Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
<b>Metals (mg/kg or ppm)</b>					
Antimony	200	9.3	150	NA	NA
Arsenic	450	<b>130</b>	57	700	700
Cadmium	14	2.7	<b>5.1</b>	9.6	9.6
Chromium	>1100	>96	260	NA	NA
Copper	1,300	390	530	390	390
Lead	1,200	<b>430</b>	<b>450</b>	530	660
Mercury	2.3	1.4	2.1	0.41	0.59
Nickel	>370	110	>140	NA	NA
Silver	6.1	8.4	>6.1	>.56	>0.56
Zinc	3,800	<b>460</b>	410	1,600	1,600
<b>Organic Compounds (ug/kg or ppb)</b>					
<b>Low molecular weight PAH</b>					
LPAH	29,000	1,200	13,000	<b>5,200</b>	5,200
2-Methylnaphthalene	1,900	64	1,400	<b>670</b>	<b>670</b>
Acenaphthene	2,000	130	730	<b>500</b>	<b>500</b>
Acenaphthylene	1,300	71	<b>1,300</b>	>560	>560
Anthracene	13,000	280	4,400	<b>960</b>	<b>960</b>
Fluorene	3,600	120	1,000	<b>540</b>	<b>540</b>
Naphthalene	2,400	230	2,700	<b>2,100</b>	<b>2,100</b>
Phenanthrene	21,000	660	5,400	<b>1,500</b>	<b>1,500</b>
<b>High molecular weight PAH</b>					
HPAH	69,000	7,900	69,000	<b>12,000</b>	17,000
Benz[a]anthracene	5,100	960	5,100	<b>1,300</b>	1,600
Benzo[a]pyrene	3,500	1,100	3,600	<b>1,600</b>	<b>1,600</b>
Benzo[ghi]perylene	3,200	920	2,600	670	720
Benzo[fluoranthene]	9,100	1,800	9,900	<b>3,200</b>	3,600
Chrysene	21,000	950	9,200	<b>1,400</b>	2,800
Dibenzo(a,h)anthracene	1,900	<b>240</b>	970	230	230
Fluoranthene	30,000	1,300	24,000	<b>1,700</b>	2,500
Indeno(1,2,3-c,d)pyrene	4,400	760	2,600	600	690
Pyrene	16,000	2,400	16,000	<b>2,600</b>	3,300

**Table 9. The 1994 suite of dry weight-normalized Puget Sound AETs.**

1994 amphipod mortality and echinoderm larvae abnormality AETs are combined with 1986 benthic abundance, 1986 Microtox luminosity and oyster abnormality AETs. A ">" symbol indicates a minimum value, not confirmed by a "Hit" sample with a higher concentration. New and confirmed HAETs are in boxes, 2AETs are in bold italics, and LAETs are shaded.

Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
<b>Chlorinated organic compounds</b>					
1,2,4-trichlorobenzene	51	>4.8	NA	31	64
1,2-dichlorobenzene	>110	NA	50	35	50
1,3-dichlorobenzene	>170	>4.4	>170	>170	>170
1,4-dichlorobenzene	120	NA	110	110	120
Hexachlorobenzene	130	NA	22	70	230
<b>Phthalates</b>					
Bis[2-ethylhexyl] phthalate	>8,300	<b>1,700</b>	1,300	1,900	1,900
Butyl benzyl phthalate	970	<b>200</b>	900	63	>470
Di-n-butyl phthalate	1,400	>31	>5100	1,400	1,400
Di-n-octyl phthalate	>2,100	>98	6,200	NA	>420
Diethylphthalate	>1,200	>62	200	>48	>73
Dimethylphthalate	>1,400	<b>85</b>	>1400	71	160
<b>Phenols</b>					
2-methyl phenol	77	55	72	>72	63
2,4-dimethyl phenol	77	55	210	29	29
4-methylphenol	3,600	110	1,800	<b>670</b>	<b>670</b>
Pentachlorophenol	400	>150	690	>140	>140
Phenol	1,200	>220	1,200	1,200	420
<b>Miscellaneous Extractables</b>					
Benzyl alcohol	73	>12	870	57	73
Benzoic acid	760	>31	650	650	650
Dibenzofuran	1,700	110	700	<b>540</b>	<b>540</b>
Hexachlorobutadiene	180	1.3	11	120	270
Hexachloroethane	140	NA	NA	NA	NA
N-nitrosodiphenylamine	48	>25	28	40	130
<b>Volatile organics</b>					
Ethylbenzene	50	4.0	10	33	37
Tetrachloroethene	>210	>1	57	140	140
Xylene, Total	160	>21	40	100	120

**Table 9. The 1994 suite of dry weight-normalized Puget Sound AETs.**

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Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
<b>Pesticides and PCBs</b>					
Aldrin	9.5	9.5	NA	NA	NA
Chlordane	2.8	>4.5	NA	NA	NA
Dieldrin	3.5	1.9	NA	NA	NA
Heptachlor	1.5	2.0	NA	NA	NA
p,p'-DDD	63	28	16	NA	NA
p,p'-DDE	62	9.3	9.0	NA	NA
p,p'-DDT	>270	12	34	NA	>6
Total DDT	24	37	NA	NA	NA
Total PCBs	3,100	450	1,000	130	1,100

**Table 10. The 1994 suite of TOC-normalized Puget Sound AETs.**

1994 amphipod mortality and echinoderm larvae abnormality AETs are combined with 1988 benthic abundance, 1986 Microtox luminosity and oyster abnormality AETs. A ">" symbol indicates a minimum value, not confirmed by a "Hit" sample with a higher concentration. New and confirmed HAETs are in boxes, 2AETs are in bold italics and LAETs are shaded.

Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
<b>Metals (mg/kg TOC or ppm)</b>					
Antimony	>15,000	>2,100	5,500	3,300	3,300
Arsenic	32,000	<b>5,800</b>	4,400	88,000	88,000
Cadmium	1,100	>430	580	1,200	1,200
Chromium	>130,000	>5,400	65,000	NA	NA
Copper	100,000	<b>30,000</b>	13,000	48,000	49,000
Lead	48,000	<b>22,000</b>	18,000	<b>66,000</b>	<b>66,000</b>
Mercury	<b>300</b>	71	120	77	210
Nickel	<b>20,000</b>	>49,000	<b>31,000</b>	NA	NA
Silver	<b>270</b>	<b>270</b>	490	100	>100
Zinc	150,000	60,000	48,000	>200,000	>200,000
<b>Nonionizable organic compounds (mg/kg TOC; ppm)</b>					
<b>Low molecular weight PAH</b>					
LPAH	2,200	22	780	>530	<b>370</b>
2-Methylnaphthalene	>120	>53	64	NA	NA
Acenaphthene	200	>110	57	>57	16
Acenaphthylene	66	>18	66	>27	>27
Anthracene	1,200	93	<b>220</b>	>79	>79
Fluorene	360	<b>73</b>	79	>71	23
Naphthalene	220	>190	170	>170	99
Phenanthrene	<b>840</b>	<b>140</b>	480	>160	120
<b>High molecular weight PAH</b>					
HPAH	5,300	150	7,600	1,500	<b>960</b>
Benz[a]anthracene	270	<b>170</b>	650	>160	110
Benzo[a]pyrene	<b>210</b>	<b>230</b>	>1300	>140	99
Benzo[ghi]perylene	<b>100</b>	>240	>1200	>67	31
Benzofluoranthenes	450	<b>310</b>	1,500	>430	230
Chrysene	840	<b>220</b>	850	>200	110
Dibenzo(a,h)anthracene	50	<b>48</b>	89	33	120
Fluoranthene	3,000	<b>320</b>	1,200	>190	160
Indeno(1,2,3-c,d)pyrene	<b>120</b>	>190	900	>87	33
Pyrene	<b>1,000</b>	520	1,400	>210	>210

**Table 10. The 1994 suite of TOC-normalized Puget Sound AETs.**

1994 amphipod mortality and echinoderm larvae abnormality AETs are combined with 1988 benthic abundance, 1986 Microtox luminosity and oyster abnormality AETs. A ">" symbol indicates a minimum value, not confirmed by a "Hit" sample with a higher concentration. New and confirmed HAETs are in boxes, 2AETs are in bold italics and LAETs are shaded.

Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
<b>Chlorinated organic compounds</b>					
1,2,4-trichlorobenzene	1.8	>2.4	NA	0.81	2.7
1,2-dichlorobenzene	>5.8	NA	2.3	2.3	2.3
1,3-dichlorobenzene	>15	>0.26	>15	>15	>15
1,4-dichlorobenzene	9	NA	16	>16	3.1
Hexachlorobenzene	4.5	NA	0.38	2.3	9.6
<b>Phthalates</b>					
Bis[2-ethylhexyl] phthalate	>550	130	60	47	60
Butyl benzyl phthalate	49	5.2	64	4.9	>9.2
Di-n-butyl phthalate	260	0.88	1,700	220	260
Di-n-octyl phthalate	58	>57	4,500	NA	>57
Diethylphthalate	>110	>0.27	61	>5.3	>5.3
Dimethylphthalate	53	NA	53	>19	>22
<b>Miscellaneous Extractables</b>					
Dibenzofuran	>170	57	58	>58	15
Hexachlorobutadiene	6.2	NA	6.9	3.9	11
Hexachloroethane	2.7	NA	NA	NA	NA
N-nitrosodiphenylamine	>11	>6.4	11	>11	>11
<b>Volatile organics</b>					
Ethylbenzene	>3.8	NA	>3.8	>3.8	>3.8
Tetrachloroethene	>22	NA	>22	>22	>22
Xylene, Total	>12	0.15	>12	>12	>12
<b>Pesticides and PCBs</b>					
Aldrin	0.56	>0.56	NA	NA	NA
Chlordane	0.16	>0.26	NA	NA	NA
Dieldrin	0.13	0.28	NA	NA	NA
Heptachlor	>0.11	>0.40	NA	NA	NA
p,p'-DDD	3.1	1.6	1.0	NA	NA
p,p'-DDE	6.0	>7.3	0.31	NA	NA
p,p'-DDT	16	>0.71	3.7	NA	NA
Total DDT	1.4	8.8	NA	NA	NA
Total PCBs	190	18	65	12	>46

**Table 10. The 1994 suite of TOC-normalized Puget Sound AETs.**

1994 amphipod mortality and echinoderm larvae abnormality AETs are combined with 1988 benthic abundance, 1986 Microtox luminosity and oyster abnormality AETs. A ">" symbol indicates a minimum value, not confirmed by a "Hit" sample with a higher concentration. New and confirmed HAETs are in boxes, 2AETs are in bold italics and LAETs are shaded.

Chemical Group/ Chemical of Concern	Amphipod AET (1994)	Echinoderm AET (1994)	Benthic AET (1988)	Microtox AET (1986)	Oyster AET (1986)
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**Ionizable organic compounds (mg/kg TOC or ppm)**

**Phenols**

2-methyl phenol	3.1	>2.1	10	>10	3.1
2,4-dimethyl phenol	6.5	NA	2.6	0.63	>1.3
4-methylphenol	780	4.7	250	81	37
Pentachlorophenol	24	>9.3	66	>11	>11
Phenol	>440	3.2	>140	33	>39

**Miscellaneous Extractables**

Benzyl alcohol	5.0	>0.71	>73	5.0	5.0
Benzoic acid	>170	>2.0	>170	>170	>170

**Table 11.** Summary of the highest, second lowest and lowest AET values (HAET, 2AET, LAET, respectively) among the suite of five 1994 Puget Sound AETs. NA = data not available.

AET Rank	Dry Weight Normalized		TOC Normalized	
	Higher	Lower	Higher	Lower
New HAETs	17	0	10	0
New 2AETs	NA	<29	NA	<24
New LAETs	0	23	0	6

The number of new 2AET and LAET values was even more pronounced than the number of HAETs which changed between 1988 and 1994. This was caused primarily by the addition of a new echinoderm AET group to the suite of Puget Sound AETs. As already discussed, the 1994 echinoderm larval abnormality AETs tended to be lower than the corresponding 1986 oyster AET values.

Lower 2AET and LAET values could have potentially greater effects on the SMS program. However, this would assume, of course, the same relationship of 2AET and LAET to those criteria and that predictive reliability of 1994 values was judged adequate.

### Reliability of 1994 Puget Sound AETs

There are four possible outcomes from comparisons of synoptic sediment quality data to AET values:

- correct prediction of samples exhibiting significant adverse effects (the chemistry of "Hit" samples was greater than AET values)
- correct prediction of samples with no significant adverse effects (the chemistry of "No Hit" samples was below AET values)
- incorrect prediction of samples exhibiting significant adverse effects (the chemistry of "Hit" samples was below AET values)

- incorrect prediction of samples with no significant adverse effects (the chemistry of "No Hit" samples was greater than AET values)

Comparisons yielding a high frequency of the first outcome involve sediment quality values, AETs or PSDDA SLs for example, which are highly sensitive. A high combined frequency of the first and second reflect AETs which have good overall reliability. The third and fourth outcomes translate to sediment quality values having low sensitivity and efficiency, respectively.

Reliability calculations showed the sensitivity of 1994 dry weight- and TOC- normalized amphipod AETs to be lower than in 1988. However, that result was predicted. As the 1994 amphipod AET values increased, the chemistry of fewer samples exceeded them, and fewer of the "Hit" samples were correctly predicted. The new amphipod AETs were particularly poor at predicting "Hit" samples from the EIGHTBAY, HULB90FC and PSDDA1 surveys (see Tables C-5 and C-6).

As described in the Results section, elevated levels of conventional parameters could have contributed to the adverse effects observed in those surveys. However, PSDDA agencies chose not to exclude incorrectly predicted "Hit" samples on that basis alone. If a stronger case could be made for doing so, or for correcting some "Hit" determinations based on high percent fines content, then the sensitivity of the 1994 amphipod AETs could increase approximately 10%. That would result in sensitivities similar to the 1988 dry weight- and TOC-normalized amphipod AET sensitivity values, but still lower than that of other AETs.

Another cause of low sensitivity could be that the 1994 amphipod AETs are simply too high. A weakness of AET-based sediment quality values is that a single synoptic sample sets each threshold value. For a variety of reasons, that sample may have a much higher concentration than the next highest "No Hit" sample<sup>18</sup>. That is also why chemically anomalous samples were excluded from 1988 amphipod and benthic AETs. It is possible, though, that a statistical test for outliers, such as the one proposed by Dixon (27), would result in more exclusions. Removal of additional high "No Hit" samples from the 1994 biological effects data would, in turn, result in lower and more sensitive amphipod AETs.

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<sup>18</sup> Among reasons for a more contaminated sediment sample not showing significant adverse benthic or bioassay effects are: low bioavailability of contaminants or ameliorating interactive effects among chemicals of concern and other sediment constituents.

The efficiency of 1994 dry weight-normalized amphipod AETs also appeared to decline. However, the overall reliability of both the new dry weight- and TOC-normalized amphipod AETs was the same as in 1988.

Pooled reliability analysis revealed the 1994 amphipod AET to be the least sensitive of the five Puget Sound AETs, but the most efficient one. Thus, the strength of the new amphipod AET values may not be in detection of all samples having adverse biological effects. Their strength may instead lie in the accuracy with which "Hit" sample predictions are made: nearly 90%. A sample determined to be a "Hit" sample based on amphipod bioassay results could be weighted more heavily in making regulatory decisions than a "Hit" sample based on other types of biological effects.

Regarding echinoderm larval AETs, the PSDDA agencies could not predict the effect of adding a new AET type to the suite of Puget Sound AETs. Would they be more or less reliable as a group? How would they contribute to the overall reliability of the suite of Puget Sound AETs?

Results indicated the dry weight-normalized echinoderm AETs were far less sensitive than 1986 oyster AETs: 48% vs. 88%. However, the independent reliability calculations indicated they were substantially more efficient and had better overall reliability than the oyster AETs. When biological effects data were pooled, the dry weight-normalized echinoderm AETs were found to have intermediate sensitivity; Microtox and oyster were more sensitive, while benthic and amphipod AETs were less so. Echinoderm AETs were the second most efficient of all AET types.

Certain other reliability analyses may still be recommended by experts or members of a Regulatory Work Group prior to use of new AET values in regulatory programs. Reliability calculations in which single chemical substitutions are made may indicate that changing an individual guideline value or standard has no effect on reliability. For the PSDDA program, comparing the various measures of reliability for current ML and SL values to the reliability of proposed new guidelines would be helpful to technical experts and policy makers. The reliability of "mixed-normalization" AETs<sup>19</sup> and independent reliability calculations for TOC-normalized AETs<sup>20</sup> may prove useful in evaluating new AET values for the SMS program. Such calculations, along with many of those suggested in Table 7, were outside the scope of this report.

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<sup>19</sup> A group of AETs which has "mixed-normalization" contains dry weight-normalized trace metal and polar organic AETs, combined with TOC-normalized nonpolar organic AETs.

<sup>20</sup> That calculation cannot presently be calculated by SEDQUAL.

## Summary

The Puget Sound sediment quality values database greatly increased in size, and perhaps in its ability to represent the whole of Puget Sound, between 1988 and 1994. However, the effort that was required to identify, obtain, quality assure, and enter significant additional synoptic data into Ecology's SEDQUAL database was substantial.

A comprehensive annual recalculation of new AETs, reliability values and their potential implications to the PSDDA and SMS regulatory programs would appear to be impractical. Given the current resource levels and priorities of the PSDDA agencies, an annual re-evaluation would require a greatly streamlined, new process. The alternative would be to plan for AET re-evaluations at less frequent intervals, perhaps every three years.

Extensive new synoptic biological sample data were analyzed for significant adverse effects, but a large number of those samples lacked an adequate reference sample to which test sample effects could be compared. Future surveys need to emphasize collection of adequate single or multiple reference area samples.

The biological effects sample data obtained since 1988 were combined with data from the 1988 sediment quality values database, and used to calculate new AET values for Puget Sound. Results showed many more of the echinoderm larval abnormality AETs represented new AET values than did the 1994 amphipod mortality AETs. The latter set 17 dry weight-normalized HAET values of potential use to the PSDDA program in revising its ML and SL guidelines. Echinoderm AETs were much more frequently either the new second lowest AET or LAET among the suite of what has become five Puget Sound AETs. That indicated that there were more potential changes to the SMS standards and program.

Changes to guidelines or standards in either program will involve review of the AET reliability results presented in this report, as well as others yet to be conducted. Values eventually adopted by either the PSDDA or SMS program will likely not compromise the reliability of current regulatory values. The practical and economic implications of any changes to PSDDA MLs and SLs, and SMS sediment quality criteria, would also need to be considered prior to adoption.

## RECOMMENDATIONS FOR FUTURE WORK

The PSDDA agencies remain committed to conducting additional technical analyses needed in order to finalize the 1994 calculations of AET and reliability values presented in this report. These analyses include:

- ▶ calculating reliability of "mixed" dry weight- and TOC-normalized 1994 amphipod mortality and echinoderm larval abnormality AETs
- ▶ performing independent reliability analysis for 1994 AETs and comparing the results to 1988 reliability values, where this has not been done
- ▶ calculating additional "pooled" reliability analyses
- ▶ calculating the effect of individual new AETs on the reliability measures for AET groups<sup>21</sup>
- ▶ conducting other analyses to determine the quality of new 1994 AET values

Some longer-term tasks which the PSDDA agencies might consider undertaking in the future include:

- ▶ examining synoptic data only available since completing the calculations presented in this report, which might affect 1994 AET values
- ▶ consider using alternate statistical methods for comparing test sediment samples to reference sediment samples and determining significance of adverse effects
- ▶ using an established statistical test for "outliers" to detect and exclude chemically anomalous samples
- ▶ calculating AET and reliability values for subregions within Puget Sound, such as Commencement Bay, Eagle Harbor, Elliott Bay or Sinclair Inlet
- ▶ calculating new juvenile polychaete growth AETs and their reliability
- ▶ calculating new AETs based on benthic infaunal effects, and the reliability of those AETs
- ▶ performing "pooled" AET analyses which include new benthic and juvenile polychaete AETs
- ▶ examining other means of calculating empirical and probabilistic effects thresholds

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<sup>21</sup> An example of this would be to calculate the pooled reliability of the 1988 suite of AETs, substitute a single new dry weight-normalized amphipod mortality AET (e.g., the benzo(a)pyrene value of 3500 ppb dry weight), recalculate the reliability, and compare the results.

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## GLOSSARY

This glossary contains operational definitions for many of the technical terms used in this report. Definitions for terms in italics were taken in part or entirely from the 1988 "Update and Evaluation of Puget Sound AET" (4).

### Abnormal, Abnormality

Larvae used in sediment bioassays are abnormal if they fail to transform into a clearly defined pluteus having a pair of well-developed arms and a second pair of arms which are just budding.

### *Adverse Biological Effect, Adverse Effect*

Any change in a biological system that results in injury or damage to an organism, population or community (e.g., death, failure to develop properly, reduced population abundance).

### *Amphipod*

A small, shrimp-like crustacean often used in laboratory bioassays to test toxicity of sediments. Puget Sound amphipod AETs are derived from bioassays using the species *Rhepoxinius abronius*, although other species are allowed.

### *Anomalous*

An unusual observation, unexpected result or localized phenomenon.

### *Anomalous Sample, Biologically or Chemically*

A sample exhibiting a significant adverse biological effect, but with chemistry which does not predict one. This may be caused by interactive toxic effects among chemicals, toxic effects of unmeasured chemicals, or Type I error in the statistical test for significant adverse effects.

Or,

A sample not exhibiting a significant adverse biological effect, but with chemistry which predicts one. This may be caused toxic chemicals which are less bioavailable than in the samples used as the basis for prediction (e.g., setting the AET). It may also occur because of high biological variability, which results in inadequate statistical power to detect significant adverse effects.

*Apparent Effects Threshold (AET)*

The sediment concentration of various chemicals of concern above which statistically significant ( $p < 0.05$ ) adverse biological effects (relative to an appropriate reference condition) are always expected. Theoretically, an AET can be calculated for any chemical and biological indicator.

*Benthic Infauna (Abundance)*

Invertebrate organisms whose life cycle takes place largely within the bottom sediments of aquatic systems. Benthic AETs for Puget Sound are based on the abundance of certain major taxonomic groups of benthic organisms.

*Bioassay*

A laboratory test used to evaluate the toxicity of a material (commonly sediments or wastewater) by measuring behavioral, physiological, or lethal responses of organisms.

*Bivalve*

Any two-shelled mollusk which, as adults, usually living in association with the bottom of various waterbodies. Larval stages of bivalves are often used in laboratory bioassays to test toxicity of sediments. Puget Sound amphipod AETs are derived from bioassays using the species *Crassostrea gigas* or *Mytilus edulis*.

*Contaminant*

A chemical or biological substance in a form or quantity which can harm aquatic organisms, consumers of aquatic organisms or users of aquatic habitats.

*Contaminated Sediment*

A sediment which contains measurable levels of contaminants.

*Control, Control Sample*

*Negative Control*

A separate bioassay sample intended to distinguish between the effects on target organisms of physical or other test conditions and the effects of exposure to chemical toxicants of concern.

"Control sediment sample..." is a "...surface sediment sample which is relatively free of contamination and is physically and chemically characteristic of the area from which bioassay test animals are collected"

(PSWQA, 1991. Puget Sound Water Quality Management Plan, Element P2).

## Control, Control Sample (cont'd)

### Positive or Reference Toxicant Control

A series of separate bioassay samples intended to show that the test organisms are appropriately sensitive to a single toxicant over a range of environmental concentrations. See also EC<sub>50</sub>.

### *Conventional Parameters*

Routinely measured sediment variables and characteristics which may cause toxicity in laboratory tests or explain unusual field observations. Examples of conventional pollutants or "conventionals" include bulk ammonia, fine grained sediment (percent clay or fines), dissolved oxygen, pH, salinity, sulfides, temperature, total organic carbon, total volatile solids and others.

### *Detection Limit*

The lowest concentration at which the presence of a particular chemical can be reliably established by a specified analytical protocol.

### Dose Response, Dose Responsive

Any relationship in which some measure of biological toxicity increases in proportion to the increasing concentration of a known contaminant or toxicant.

### *Dredged Material*

Sediments excavated from the bottom of a waterway or water body.

### EC<sub>50</sub>, Effective Concentration - 50%

The concentration of a known toxicant which causes a specified effect in 50% of test organisms.

### Echinoderm

Any member of the Class Echinodermata, including sand dollars, sea stars, sea urchins, etc. Larval stages of echinoderms are often used in laboratory bioassays to test toxicity of sediments. Puget Sound echinoderm AETs are derived from bioassays using the species *Dendraster excenticus* and *Strongylocentrotus purpuratus*.

### *Efficiency*

The proportion of all samples predicted to exhibit significant adverse biological effects which actually do exhibit them.

#### Endpoint

The indicator of adverse biological effects or toxicity which is evaluated upon termination of sediment bioassays. In this report, "endpoint" does not refer to the duration of the bioassay, i.e., the point in time at which a test is terminated.

#### Equilibrium Partitioning, Equilibrium Partitioning Coefficient (EqP)

#### HAET

The highest of the four types of 1988 Puget Sound AET values or the highest of the five 1994 Puget Sound AETs for a given chemical of concern. Used to set many of the PSDDA program maximum level (ML) guidelines.

#### Hit, Hit Sample

A significant ( $p < 0.05$ ) adverse biological effect or a sample exhibiting one.

#### *Impacted Site, Station*

A site or station having significant adverse biological effect(s).

#### Independent reliability (analysis or calculation)

The calculation consists of 1) temporarily withdrawing a single sample from a data set, 2) computing AET values from the samples remaining, 3) comparing the single sample to those AETs, 4) recording whether the comparison predicted the sample which was temporarily withdrawn to be a "Hit" or a "No Hit" sample, 5) recording whether or not that prediction was correct, and 6) repeating the process for all remaining samples (withdrawing a single sample, calculating AETs, comparing the single sample to those AETs, and so on). Efficiency is calculated in the usual manner, but is no longer 100% by definition. Therefore, this procedure allows a meaningful comparison of efficiency values among different groups of AET values.

#### LAET

The lowest of the four types of 1988 Puget Sound AET values or the lowest highest of the five 1994 Puget Sound AETs for a given chemical of concern. Used to set some of the PSDDA program screening level (SL) guidelines and most of Washington's Sediment Quality Standards (SQS).

#### LC<sub>50</sub>, Lethal Concentration 50%

The concentration of a known toxicant which causes 50% mortality in test organisms.

*Maximum Level (ML)*

A sediment quality value based on the highest AET among a range of biological indicators. ML values are used in the PSDDA program to evaluate when biological testing may not be necessary to determine that a contaminated sediment is unsuitable for unconfined, open-water disposal.

*Metals, Trace*

Naturally occurring elements which, when present in the environment at unnatural levels, can be toxic. Some examples include cadmium, chromium, copper, lead, mercury, nickel, silver and zinc.

*Microtox<sup>®</sup>*

A laboratory test using light emitted by luminescent bacteria (*Photobacterium phosphoreum*) to assess the toxicity of saline or organic extracts of sediments, or of the sediments themselves.

*Mortality*

An indicator of adverse or toxic effects in various bioassays. It is usually calculated from a count of the total or total normal organisms surviving at the end of the test period.

*Nonimpacted*

Not affected adversely by human activities.

*Organic Carbon*

Carbon derived from living organisms.

*Polychaete*

Any of member of the annelid family Polychaeta. Typically a bristly marine, bottom-dwelling worm. Juvenile polychaetes, in a rapid phase of growth, are often used in laboratory bioassays to test toxicity of sediments. Puget Sound polychaete AETs, not yet calculated, will derived from bioassays using the species *Neanthes arenaceolata*.

*Polychlorinated Biphenyls*

A group of manmade organic chemicals, including 209 different but closely related compounds (congeners) made of carbon, hydrogen and chlorine. If released to the environment, they persist for long periods of time and can enter food chains.

*Pooled reliability*

The reliability of an AET or other sediment quality values group in which a sample is considered a "Hit" if it exhibits *any* significant adverse biological effect.

### *Power Analysis*

A mathematical technique to determine the capacity of a statistical test to detect true differences between treatments (e.g., between amphipod mortality measured in a test sediment and that measured in a reference sediment).

### Quality Assurance

The general process by which the precision and accuracy of (environmental) data are assessed.

### QA1

**Quality Assurance, Level 1.** The level of quality assurance which includes independent evaluation of the results from various quality control samples. Those samples include laboratory blanks and duplicates, matrix (sediment) and/or surrogate spikes, and standard reference materials. Sediment management programs use data meeting QA1 requirements to make regulatory decisions.

### QA2

**Quality Assurance, Level 2.** The level of quality assurance which includes independent evaluation of instrument calibration records, review of actual instrument outputs (paper or tape), and recalculation of final test sample results by for 10% of all samples. Sediment management programs use data meeting QA2 requirements to calculate or revise regulatory guidelines or standards.

### Reference Area

" ... an area believed to be free from chemical contamination ... "

### Reference Sample, Reference

A "reference sediment sample ... " is a " ... surface sediment sample which serves as a laboratory indicator of a test animal's tolerance to important natural physical and chemical characteristics of the sediment ... "  
(18)

"...represent the non-anthropogenically affected background surface sediment quality of the sediment sample."

'Where physical/chemical characteristics of control sediments differ from test sediments, reference sediment samples must be run.'

reference samples must be "...collected from an area believed to be free from chemical contamination..."

## Reference Sample, Reference (continued)

reference area "sediments should be relatively clean."

### *Reliability, or Predictive Reliability*

The ability of a given AET or other sediment quality values group to correctly predict significant adverse biological effects, or the lack thereof. Three measures of reliability are sensitivity, efficiency and overall reliability.

### Sample

A volume of sediment collected from one station using any of a variety of devices. A single sediment sample can be collected from multiple lowerings and retrievals of that device, if all the sediment is homogenized before being placed in sample containers. If there is no homogenization from lowering and retrieval, then field replicates are collected.

### *Screening Level (SL) Concentration or Guideline*

A sediment quality value which is based on the highest of the suite of Puget Sound AETs, but modified with a 1/10 safety factor (HAET/10). SLs are used in the PSDDA program to evaluate when biological testing should be conducted to determine the suitability of sediment for unconfined, open-water disposal.

### *Sediment*

Material suspended in or settling to the bottom of a water body, such as sand and mud which make up much of the shorelines and bottom of the Puget Sound. Sediment comes from natural sources (e.g., soil erosion) and anthropogenic sources (e.g., construction practices). Certain contaminants tend to collect on or adhere to sediment particles. Puget Sound sediments may contain elevated levels of some contaminants relative to reference areas located away from major contaminant sources.

### *Sediment Quality Value*

Any of ... A "benchmark" number for an environmentally acceptable concentration of a given chemical in sediments. A chemical concentration that is expected to be below the level that would consistently lead to adverse biological effects for a wide variety of species, habitats, and sediment types.

### SEDQUAL

Sediment quality database and menu-driven program developed for the Puget Sound Estuary Program and used by Ecology to calculate sediment

quality values, evaluate the suitability of dredged material for disposal in open water, assess the need for sediment cleanup, etc. Also, the "AET database."

*Sensitivity*

The proportion of all sediment samples exhibiting a significant adverse biological effect which are correctly predicted by a sediment quality value, e.g., AET.

*Station*

Any physical location, unique in space and time, at which sediment (or other) samples are collected. Station locations are usually stored as degrees, minutes and decimal seconds of latitude and longitude, along with water depth.

*Statistically Significant*

A quantitative determination of the statistical degree to which multiple measurements of the same variable can be shown to be different, given the variability of the measurement.

*Survey*

Any field investigation involving collection of (marine sediment) samples for a single identified purpose, whether that be related to dredging, monitoring, reconnaissance, remediation or scientific study. Surveys may be completed in a single day or may be conducted over many weeks or months.

*Synoptic Data*

Chemistry and bioassay data representing subsamples taken from the same homogenized sediment sample, collected on the same day, as part of the same sampling effort.

*Synoptic Survey*

Any survey which collects synoptic data.

*Toxicant*

Any man-made or naturally-occurring chemical which causes acute, chronic or other adverse effects in living organisms.

# *APPENDIX A*

## *METHODS*

## Contents

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## AMPHIPOD SEDIMENT BIOASSAY

### USE AND LIMITATIONS

The amphipod (*Rhepoxynius abronius* 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. 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 *R. abronius* bioassay is appropriate for sediments with interstitial water salinity of  $\geq 25$  ppt. The *E. estuarius* bioassay is appropriate for sediments with interstitial water salinity of 2-28 ppt. In addition, the following constraints apply:

- For the *R. abronius* bioassay, an interstitial water salinity of  $\geq 25$  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* bioassay is preferred for sediments with salinities  $< 25$  ppt.
- Grain size may have an effect on the animals at extremes of fine and coarse material (DeWitt et al. 1988). If the clay content of the test sediments exceeds 50 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 (Swartz et al. 1985).
- 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).

**Exhibit A-1**  
**Laboratory Sediment Bioassays**  
**Amphipod Bioassay**  
**Revised July 1991**

- Identification of *R. abronius* 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).

These guidelines have been adapted from Swartz et al. (1985) for *R. abronius* and DeWitt et al. (1989) for *E. estuarius*. A new 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 species of amphipods 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. 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 found in intertidal estuarine sediments from +0.5 meters to +2.0 meters above mean lower low water. 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 beaker 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.

### **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. Amphipods 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. 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.

**Sediment**—Control, reference, and test sediments should be stored at 4°C in the dark. Holding time should not exceed 14 days.

## LABORATORY PROCEDURES

The laboratory procedures 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
- Sediment holding time prior to testing is set at a maximum of 14 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.

### Test Animals

**Sieving**—A 1.0-mm sieve is used to remove *R. abronius* and *E. estuarius* from sediment. 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.

**Sorting**—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 28 ppt seawater 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. 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. Amphipods should be acclimated to laboratory conditions for a minimum of 2 days and a maximum of 10 days before testing.

The identification of amphipods as *R. abronius* or *E. estuarius* must be confirmed by a qualified taxonomist. In addition, representative specimens from each bioassay series should be preserved and archived for future reference.

## Control Sediment

*R. abronius* and *E. estuarius* typically inhabit well-sorted, fine sand. Suggestions for sieving and settling may have to be adjusted for other sediment types.

Approximately 0.25 liters of control sediment should be collected for each bioassay beaker. 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*, ambient salinity for *E. estuarius*). 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 beaker. 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, interstitial water salinities below 25 ppt may require adjustment upwards. Both *R. abronius* and *E. estuarius* are tolerant of a range of sediment grain size. However, controls for the effects of particle size distribution are recommended if the silt and clay content exceeds 50 percent or the gravel content exceeds 35 percent.

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. Adjustment of interstitial water salinity is appropriate only for dredged material destined for marine disposal. The following procedure is recommended in such cases. 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  $28 \pm 1$  ppt for the *R. abronius* test and at ambient interstitial salinity for the sediment collection site for the *E. estuarius* test. Temperature of seawater

used in bioassays of either species is maintained at  $15 \pm 1^\circ\text{C}$ . The bioassay seawater must be uncontaminated, which may necessitate collection of seawater at the amphipod collection site. Natural and reconstituted seawater should be held at  $\leq 15^\circ\text{C}$  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  $0.45\text{-}\mu\text{m}$  pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water 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. Beakers are filled to 750 mL with seawater at 28 ppt for the *R. abronius* test or ambient interstitial salinity for the *E. estuarius* test, covered with a watchglass, and placed in a  $15^\circ\text{C}$  water bath. Constant illumination is provided by overhead lights. Water in the beakers 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 beaker 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 failure to rebury in sediment at the end of the exposure period.

**Initiation**—The day before the bioassay is initiated, each test sediment sample is homogenized and an aliquot sufficient to make a 2-cm-deep layer is added to a bioassay beaker. For replicate bioassay samples, the weight of sediment necessary to make a 2-cm-deep layer (approximately 175 mL) in the first beaker is added to

the other replicates. The same procedure applies to control sediment. Treatments are randomly assigned to prenumbered bioassay beakers.

The sediment aliquot in the beaker 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 beakers. This disk is removed and rinsed in bioassay water between beakers and changed between treatments. The beakers are covered with watchglasses, put into the 15°C water bath or temperature-controlled room, and aerated. The beakers 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 beakers must be set up for this purpose. Monitoring the quality of seawater overlying the sediment can be accomplished in the bioassay beakers without disturbing the sediment. Temperature is recorded from a thermometer maintained in a separate beaker containing control sediment and bioassay water but no amphipods.

On the day the bioassay is initiated, amphipods are distributed among all beakers so that each receives 20 individuals. It is usually not logistically possible to distribute amphipods to all beakers at the same time, so it is necessary to select a portion of the beakers (as many as 15) to be processed together. The exact number of beakers 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 1.0-mm sieve, 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 beakers. 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 beakers 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. 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.

**Monitoring**—If samples for chemical analysis are desired, seawater and sediment samples can be taken from beakers at the initiation of the bioassay. A small quantity of seawater can be taken from beakers 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 beaker set up for this purpose is monitored. Lighting and aeration systems are checked. Each beaker 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 beakers 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.

## Experimental Design

**Logistics**—A typical sediment bioassay involves about 50–60 bioassay beakers. 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 beakers 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  $\leq 10$  percent and individual replicate mortality should be  $\leq 20$  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 (contaminated) control is also required for all testing. This involves determining 96-hour  $LC_{50}$  values for *R. abronius* 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. Swartz et al. (1986) determined a 96-hour  $LC_{50}$  of 1.61 mg/L for cadmium chloride. Acute lethality results for a reference toxicant must be reported along with the sediment bioassay results. Bioassays to establish

an LC<sub>50</sub> involve four or five logarithmic concentration series and a control. At least one treatment should give a partial response below the LC<sub>50</sub> and one above the LC<sub>50</sub>. Statistical procedures for the LC<sub>50</sub> 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 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. 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 beakers. 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 beaker and the 10-day mean and standard deviation for each treatment

**Exhibit A-1**  
**Laboratory Sediment Bioassays**  
**Amphipod Bioassay**  
**Revised July 1991**

- Failure to rebury (optional) for each beaker and the mean and standard deviation for each treatment
- 10-day mortality and total effective mortality (optional) in each beaker 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.

**Exhibit A-1**  
**Laboratory Sediment Bioassays**  
**Amphipod Bioassay**  
**Revised July 1991**

## 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 are not associated with the types of sediments that generally are tested using this method. 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 recommended. This should comprise 48-hour LC<sub>50</sub> and EC<sub>50</sub> 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 evaluated.
- 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. Accordingly, it is recommended that a few sediment samples from each set of bioassays conducted with this technique be examined to determine whether living larvae are present in the sediment. The results should be quantified and reported.

## 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 20 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 not exceed 14 days.

## 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 prior to testing is set at a maximum of 14 days

- Seawater holding time prior to testing is set at a maximum of 2 days for field-collected and reconstituted seawater
- 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
- Twenty grams of sediment is suspended in 1 liter 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
- Aeration is specified for test chambers in which dissolved oxygen concentrations decline below 60 percent of saturation
- A positive control (reference toxicant) is recommended
- Additional seawater controls are added for monitoring the stage of larval development
- Minimum sample sizes of 100 larvae in each replicate control chamber and 20 larvae in each replicate test chamber are recommended for abnormality measurements
- 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. 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 ( $20 \pm 1^\circ\text{C}$  for oysters and  $16 \pm 1^\circ\text{C}$  for mussels) and salinity ( $28 \pm 1$  ppt) should be attained gradually at increments not exceeding  $2^\circ\text{C}/\text{day}$  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).

### **Bioassay 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.

### **Bioassay Seawater**

Seawater used in the bioassay is maintained at a salinity of  $28 \pm 1$  ppt and temperature of  $20 \pm 1^\circ\text{C}$  for oysters and  $16 \pm 1^\circ\text{C}$  for mussels. Seawater should be collected from uncontaminated areas (e.g., deep or offshore waters) to avoid contamination and should be held at  $\leq 20^\circ\text{C}$  for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1989) should be held at  $\leq 20^\circ\text{C}$  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 not incur more than 10-percent abnormal development or 30-percent mortality during 48 hours of exposure to the bioassay seawater.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with 0.45- $\mu\text{m}$  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, 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  $20 \pm 1^\circ\text{C}$  for oysters and  $16 \pm 1^\circ\text{C}$  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. Twenty grams (wet weight) of the appropriate sediment is added to each bottle and volume is brought up to 1 liter with filtered or UV-treated seawater ( $28 \pm 1$  ppt salinity) to make a final concentration in all containers of 20 grams (wet weight) of sediment per liter of seawater. The reference area sediment chambers each contain 20 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  $20 \pm 1^\circ\text{C}$  for oysters and  $16 \pm 1^\circ\text{C}$  for mussels 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 that chamber should be aerated gently for

**Exhibit A-1, continued**  
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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 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 (1989) 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 (1989), 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 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 test beakers, 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 if it is suspected these variables may interfere with the results for the test sediment.

**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 beakers, 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 stirred, 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 300–500 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 larvae surviving relative to the mean number of 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.

Photomicrographs or drawings that illustrate the proper interpretation of "normal" and "abnormal" development are available from U.S. Army Corps of Engineers, Seattle District, or EPA Region 10.

### 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 survive the 48-hour exposure with seawater alone, and of these at least 90 percent must show no abnormalities. 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
- 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 larval presence in the sediment
- Any problems that may have influenced data quality.

## 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. Purple sea urchins (*Strongylocentrotus purpuratus*), green sea urchins, (*S. droebachiensis*), and sand dollars (*Dendraster excentricus*) 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 may also be calculated from the number of normal surviving larvae.

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 recommended. This should comprise 48-hour LC<sub>50</sub> and EC<sub>50</sub> 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 evaluated.
- 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. Accordingly, it is recommended that a few sediment samples from each set of bioassays conducted with this technique be examined to determine whether living larvae are present in the sediment. The results should be quantified and reported.

## **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 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 20 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 not exceed 14 days.

## **LABORATORY PROCEDURES**

The following procedures are synthesized primarily from Dinnel and Stober (1985) and Dinnel and Kocan (1988). The following changes were incorporated:

- The salinity of test water is adjusted to 28 ppt
- Seawater temperature during the bioassay is maintained at  $15 \pm 1^\circ\text{C}$  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 prior to testing is set at a maximum of 14 days
- 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
- A positive control (reference toxicant) is recommended
- Additional seawater controls are added for monitoring the stage of embryo development
- Two endpoints are measured: mortality and abnormality (a combined mortality/abnormality endpoint can also be calculated)
- Minimum sample sizes of 100 larvae in each replicate control chamber and 20 larvae in each replicate test chamber are recommended for abnormality measurements.

## **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. If animals are transported in seawater, the seawater should be kept cool ( $4^\circ\text{C}$ ) and, if necessary, aerated. 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.

### **Bioassay 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  $28 \pm 1$  ppt. Water temperature should be maintained at  $15 \pm 1^\circ\text{C}$  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  $\leq 15^\circ\text{C}$  for no longer than 2 days before inoculation. Reconstituted seawater (ASTM 1989) should be held at  $\leq 15^\circ\text{C}$  for no longer than 2 days before use. The bioassay seawater must be uncontaminated and of acceptable low toxicity. The biological criterion of acceptability is that the larvae, spawned by adults in the dilution water, must not incur more than 10-percent abnormal development or 30-percent mortality during 48 hours of exposure to the bioassay seawater.

Bioassay seawater is passed through an ultraviolet sterilizer or a filter with a  $0.45\text{-}\mu\text{m}$  pore diameter. If necessary, salinity is reduced by addition of deionized distilled water or raised by addition of clean oceanic water, sea salt, or reagent-grade chemicals (ASTM 1989).

### **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 beaker (10-cm internal diameter) covered with an 11.4-cm-diameter watchglass. The beakers are maintained at  $15 \pm 1^\circ\text{C}$  in a shallow water bath, incubator, or temperature-controlled room with an overhead aeration source. General recommendations of ASTM (1989) 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 hours, during which the embryos normally will develop into the four-armed pluteus stage. A slightly longer exposure period may be used if necessary to achieve adequate development of embryos in seawater controls. Exposure time should not exceed 96 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.

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  $\leq 2,000:1$ . 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 beakers should be prepared prior to spawning of the adult echinoderms to allow enough time for sediments to settle in the test beakers 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. Twenty grams of reference or test sediment is added to each beaker. Filtered or UV-treated seawater (28 ppt salinity) is added to each beaker up to 1 liter to make a final concentration in all containers of 20 grams (wet weight) of

Exhibit A-1, continued  
Laboratory Sediment Bioassays  
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sediment per liter of seawater. Each reference area sediment chamber also contains 20 grams/liter 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 beakers 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  $15 \pm 1^\circ\text{C}$  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 that chamber 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 (1989) 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 (1989), 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 beakers, 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 if it is suspected these variables may interfere with the results for the test sediment.

**Termination**—The bioassay is terminated when greater than 95 percent of the embryos in the duplicate seawater control have reached the four-armed pluteus stage (approximately 48–96 hours). Once this stage has been achieved in the control beakers, 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 beaker are carefully poured into a clean 1-liter beaker. The water is then mixed thoroughly using a perforated plunger and 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.

**Exhibit A-1, continued**  
**Laboratory Sediment Bioassays**  
**Echinoderm Embryo Bioassay**  
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Preserved samples (equal in volume to those containing 300–500 larvae in controls) are examined in Sedgewick-Rafter cells. Normal and abnormal larvae are enumerated to determine percent survival and percent abnormality. 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. 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. Aliquot counts should be completed even after the minimum sample size is achieved. Percent survival for each replicate bioassay chamber is based on the number of larvae surviving relative to the mean number of survivors in the seawater controls. Embryos that fail to transform to the four-armed pluteus stage are considered abnormal. Percent abnormal for each replicate bioassay chamber is based on the number of survivors that are 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 survive the 48-hour exposure with seawater alone, and of these at least 90 percent must show no abnormalities. 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)  $LC_{50}$  and  $EC_{50}$  values for echinoderm 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  $LC_{50}$  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 assay:

- Source, qualitative condition, and holding time of brood stock
- All water quality measurements [e.g., dissolved oxygen, temperature, salinity, pH, sulfides (optional), and ammonia (optional)]

**Exhibit A-1, continued**  
**Laboratory Sediment Bioassays**  
**Echinoderm Embryo Bioassay**  
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- Individual replicate and mean and standard deviation data for larval percent mortality at termination of bioassay
- Individual replicate and mean and standard deviation data for larval percent abnormality at termination of bioassay (optional)
- Individual replicate and mean and standard deviation data for larval percent combined mortality plus abnormalities at termination of bioassay
- $LC_{50}$  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 larval presence in the sediment
- Any problems that may have influenced data quality.

## Exhibit A-2

### Modifications to Bioassay Protocols

#### Bioassay Protocols:

#### PSDDA Modifications to the Amphipod and Sediment Larval Tests

The following outline lists changes to the amphipod and sediment larval bioassay protocols which the PSDDA agencies have adopted or recommended since the PSEP Protocols (6) were published.

- I. 10-day amphipod mortality bioassay modifications.
  - A. Bioassay laboratories should avoid sexually dimorphic male amphipods (19).
  - B. Reference sediments should be collected subtidally and be grain size matched to test sediment samples using a wet sieving technique (8,19).
  - C. Laboratories should analyze reference sediment samples for bulk ammonia and total sulfides for 10-day amphipod mortality bioassay (8).
  - D. Laboratories should collect and report amphipod reburial data (9).
  - E. The holding time for bioassay test samples was extended to a maximum of eight weeks (9).
  - F. Under certain circumstances, *Ampelisca abdita* or *Eohaustorius estuarius* may be substituted for *Rhepoxinius abronius* (11).
  
- II. Sediment larval bioassay modifications.
  - A. Echinoderm larval bioassays should be conducted at 15°C for a minimum of 48 hours. Guidelines for initiating and terminating the test were recommended (19)
  - B. Alternate reference areas may be used, if the sediment chemistry of those areas is adequately characterized (8).
  - C. Laboratories should analyze reference sediment samples for bulk ammonia and total sulfides for echinoderm larval bioassay (8).
  - D. PSDDA adopted several changes to the test temperature, duration, endpoint and termination of the echinoderm larval bioassay protocol (9).

## Exhibit A-2

### Modifications to Bioassay Protocols

- E. The holding time for bioassay test samples was extended to a maximum of eight weeks (9).
- F. Seawater control and reference sample performance standards were revised. The seawater control abnormality standard was eliminated. The performance standard for seawater control samples was changed to  $\leq 30\%$  effective mortality (abnormality plus mortality). Reference samples should not exceed 30% effective mortality when seawater control-normalized (12).
- G. In order to increase statistical power, certain modifications were made to interpretive guidelines for the sediment larval bioassay (12).

**Exhibit A-3**  
Sediment Larvae Bioassay  
Data Entry and Response Calculation

**Sediment Larval Bioassays: Data entry and Response Calculation.**

Sediment larval bioassay laboratory results were entered in the sediment quality values database (SEDQUAL) using the same standard conventions as were used prior to calculating 1988 AETs (*PTI Environmental Services, Inc., personal communication*). Total and normal larvae surviving, both at test initiation and conclusion, were entered as described below. The percent abnormality, mortality and effective mortality (abnormality + mortality) endpoints for echinoderm and bivalve larval bioassays were then calculated from those data.

**I. Data Entry**

**A. Abnormality Endpoint.**

1. Sea water (negative) control sample.
  - a. Initial Value = the total number of survivors in each replicate (normal + abnormal) at the end of the sea water control exposure.
  - b. Final Value = the total number of normal survivors in each replicate at the end of the sea water control exposure.
2. Positive control sample.
  - a. Initial Value = the total number of survivors in each replicate (normal + abnormal) at the end of the positive control exposure.
  - b. Final Value = the total number of normal survivors in each replicate at the end of the positive control exposure.
3. Reference area and test sediment samples.
  - a. Initial Value = the total number of survivors in each replicate (normal + abnormal) at the end of the test/reference sediment sample exposure.
  - b. Final Value = the total number of normal survivors in each replicate at the end of the test/reference sample exposure.