DMMP/SMS CLARIFICATION PAPER

BIOASSAY ENDPOINT REFINEMENTS: BIVALVE LARVAL AND NEANTHES GROWTH BIOASSAYS

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INTRODUCTION/BACKGROUND

Bioassays are used in the Dredged Material Management Program (DMMP) to evaluate toxicity in sediments proposed for dredging, and in the Sediment Management Standards (SMS) Program to assess toxicity at cleanup sites. Agency decisions based on bioassay results can have significant economic and environmental consequences; therefore it is critical that these tests provide meaningful results. The DMMP and SMS have both relied on an adaptive management approach to examine and adopt improvements to bioassays. Proposed changes are presented for public review as issue or clarification papers through the Sediment Management Annual Review Meeting (SMARM) process. Since PSDDA/DMMP implementation in 1988, a total of twenty-seven changes to existing bioassay protocols having been made though the SMARM process. A complete list of these changes can be found in **Table 1**.

At the 2010 SMARM, potential method modifications to improve the performance of the juvenile *Neanthes* growth bioassay and the *Mytilus galloprovincialis* bivalve larval bioassay tests[1] were presented (Gardiner, W., 2010). Additional data to further support these method modifications were presented at the 2011 SMARM (Word, J., 2011). Several federal navigation projects have since been used as test cases, as well as results from monitoring at the DMMP disposal site in Port Gardner. Case studies from cleanup areas in Port Gamble are provided to illustrate the effects of bioassay endpoint adjustments relative to reducing false positive results.

This clarification paper summarizes the confounding factors associated with these two tests, the proposed methodological revisions to address these confounding factors and the results of side-by-side comparisons of the existing and modified protocols conducted by the DMMP and SMS programs.

PROBLEM IDENTIFICATION

The purpose of this clarification paper is to provide method modifications intended to reduce the following confounding grain-size effects in the juvenile *Neanthes* growth bioassay and the *Mytilus galloprovincialis* bivalve larval test:

[1] The DMMP agencies and SMS Program have not evaluated the effects of the resuspension endpoint protocol relative to the Sediment Echinoderm Larval Test (*Dendraster excenctricus*), but intend to evaluate this endpoint adjustment in the future to assess potential performance improvements to the sediment echinoderm larval test.

- For the *Neanthes* growth test, there is considerable variation in weights of animals at test termination due to sediments retained in the guts of test organisms being unintentionally included in the measured biomass. Organisms ingesting coarse-grained sediment have higher inorganic gut content than those grown in fine-grained sediment.

- For the bivalve larval test, the existing test-termination protocol can lead to poor recovery of normal surviving larvae, artificially elevating the toxicity ascribed to some sediment samples.

  For the bivalve larval test, these confounding factors appear to be more of a problem when testing sediments from areas with particularly high contents of clay and fine grained woody material.

<u>*Neanthes* Growth Test</u>.

<u>Background:</u> The *Neanthes* test provides a chronic measure of toxicity, evaluating the growth of worms over a 20-day exposure period (Peeler, M. 1992*)*. Juvenile worms, early in development, are placed in sediment and fed every two days to promote growth. Worms at the end of the test are dried overnight at 60^{o}C, and then weighed. The performance of reference sediments is evaluated by comparison of growth to that in the negative control sediment ($MIG_R/MIG_C > 0.80\%$)[2]. If the performance standard is met, growth in test sediments is then compared to growth in both the reference and control sediments. If the performance standard is not met, test sediments can only be compared to the control.

<u>The Problem</u>: The *Neanthes* test has been frequently subject to reference performance failures, particularly when testing sediments with a high fines content. Coarse sand is used as the negative control substrate, and retention of coarse sediments in the gut of control worms at the end of the test artificially inflates the growth rate when compared to growth in fine-grained reference and test sediments.

<u>Proposed Solution:</u> A simple method of eliminating the bias due to gut content is to use an ash-free dry-weight (AFDW)[3] endpoint, which represents the mass of biological tissue after subtracting the weight of inorganic materials present in the gut of animals being tested. It is instructive to note that the following biomass-based tests with sediment "ingesters" already use an AFDW endpoint:

- *Chironomus* growth test (Sibley et al. 1997)
- *Neanthes* growth test developed by USACE-ERDC (Bridges, T.S, J.D. Farrar 1997)

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[2] MIG = mean individual growth rate (mg/individual/day)

[3] After determining the dry weight, worms are placed in a muffle furnace @550^{o}C for two hours and the weight of the ashed residue is measured. This weight is then subtracted from the dry weight to determine the ash-free dry weight. See **Attachment 1** for more complete protocol description.

<u>Dry-weight versus AFDW performance - DMMP:</u> The DMMP agencies have conducted side-by-side testing comparing the existing Puget Sound Estuary Program protocol (based on dry weight) and the modified AFDW protocol. This testing has been performed on sediments from three federal O&M projects, including two testing rounds for the Grays Harbor Navigation Channel, and one testing round for the Duwamish Navigation Channel. It was also performed as part of DMMP monitoring at the Port Gardner disposal site in 2010. Side-by-side testing was especially pertinent for the Grays Harbor project, as reference sediment performance failures had been documented there in dredging years 2001, 2005, 2009, 2011 and 2012.

Comparison of dry-weight and AFDW measurements in worms exposed to different sediments illustrates the effects of gut-related sediment on the assessment of growth in this bioassay. **Figures 1a – d** present MIG based on dry weight and AFDW for the four DMMP studies where side-by-side testing was performed. No differences were observed between treatments and reference sediments using either dry-weight or AFDW MIG. However, a large enough difference was observed in dry-weight MIG between control and reference sediments for the two case studies in Grays Harbor for the reference to fail the performance guidelines (**Figure 1b-c**). The AFDW adjustment improved the performance, but the reference sediment still failed the performance guideline for the 2011 Gray Harbor testing, whereas the AFDW adjustment enabled the reference sediment to meet the performance guideline in the 2012 Grays Harbor testing. In general, the performance of the *Neanthes* test improved using the AFDW values as compared with the PSEP dry-weight measurements. For these four studies, coarse-grained controls were observed to have an average of 31% of the worm weight attributable to sediments in the gut of the worms, as compared to an average of 20% in reference sediments and 18% in treatment exposures (**Figures 2a-d**).

<u>Dry-weight versus AFDW performance - SMS Program</u>: Ecology evaluated the implementation of the AFDW protocol for the *Neanthes* growth test during the Port Gamble remedial investigation conducted during the summer of 2011. All 12 of these sediments passed the Neanthes growth test SQS and CSL biological standards for both dry-weight and AFDW measures (**Figure 1e**). Dry-weight MIG values were consistently higher than those based on AFDW, although the overall difference between dry weight and ash-free dry weight was slightly less than reported for the DMMP program. Coarse-grained sand in the gut contributed 21% of worm weight in the control, and an average of 21% for the three reference sediments. The mean weight contributed by gut contents for the 12 test sediments was 15% and ranged from 7% to 18%, (**Figure 2e**).

<u>Bivalve Larval Test</u>.

<u>Background:</u> The existing PSEP bivalve larval sediment bioassay provides a measurement of normal larval development in the presence of sediment. The protocol requires shaking 18 grams of sediment in 900 mL of water and allowing the suspended sediments to settle out over a four hour period. The test is initiated with non-swimming 2-hour-old embryos that develop into swimming larvae with shells. The larvae are

allowed to develop into D-shell-stage larvae. At the end of the test (~48 hours) the overlying water is gently stirred – without disturbing the sediment at the bottom of the test chamber - then decanted. Aliquots of the decanted water are collected and enumerated, with larvae scored as normal or abnormal. Developmentally-delayed larvae are counted as abnormal. Larvae that have died during testing decompose quickly and are generally not recovered.

The Problem: Observations during testing have shown that initial shaking of sediment in the water and settling prior to introducing the embryos results in a stratification of the sediment by grain size. As would be expected, the coarser material settles first, followed by the finer fractions. For some sediments this can result in a substantial layer of fine-grained material that may continue to settle during the first 12-24 hours of the test. The early non-swimming larvae can become buried or entrapped in this layer, which ultimately prevents them from swimming up into the overlying water. These entrapped larvae are then missed when the overlying water is decanted off for counting upon test termination.

Entrapment of larvae does not appear to be related to sediment chemistry. Examination of larvae recovered from the flocculent layer has shown them generally to be normal "D-shaped" larvae. Therefore, the loss of these larvae prior to enumeration biases the results.

Entrapment of larvae was recognized as an issue early in the development of this bioassay protocol and has been discussed at several workshops held by the DMMP agencies (PSDDA 1989; PSDDA 1990, DMMP 1998). The agencies subsequently attempted to resolve some of the problems with false positives during 1993 methods refinement effort (EPA, 1993), due to presence of suspended sediment in test chambers, and sensitivity to ammonia, and grain size. The results of this effort highlighted and documented the problems, but did not resolve them.

Proposed Solution: Bill Gardiner and Brian Hester at NewFields developed a laboratory protocol in 2009 with a step added to the standard PSEP protocol to address the larval entrapment issue. It involves conducting the standard PSEP larval test, but with a modified test-termination procedure. At approximately 42 hours from test initiation, the water, larvae and settled sediment are homogenized by gentle mixing using a perforated plunger. The contents are then allowed to settle until the test is terminated at the test duration indicated in the standard PSEP test method (48 to 60 hours). At test termination, the overlying water is decanted, aliquots are collected, and larvae are enumerated as in the standard protocol. This adjustment allows for the recovery of any larvae trapped in fine sediments or flocculent materials. The full protocol is described in **Attachment 1**.

Protocol Comparison - DMMP: The DMMP agencies evaluated the resuspension protocol using the same studies cited above for the *Neanthes* test (Port Gardner disposal site monitoring (2010), Grays Harbor O&M testing 2011 & 2012, and Duwamish O&M testing 2011. Generally, use of the resuspension adjustment made little difference in the results of the larval testing **Figures 3a-d**. However, none of the sediments evaluated within the four case studies had high concentrations of wood waste or fine-grained/

flocculent material. The only 1-hit response occurred in the Duwamish Waterway O&M characterization and was confirmed by both the PSEP and resuspension protocols (Sample T15 in **Figure 3d**). Of the eight samples for which 2-hit responses were observed, half were confirmed by both protocols and half scored hits under one protocol but not the other. None of these 2-hit responses were corroborated by the other bioassays in the testing suite (amphipod mortality and *Neanthes* growth).

Protocol Comparison - SMS Program. Ecology has evaluated the development and application of the resuspension protocol in recent testing of Port Gamble sediments. Outcomes were compared for 31 test sediments ranging from very fine-grained sediments with wood waste to sands with low organics (**Figure 4a-b**). The greatest increase in the number of recovered normal survivors using the resuspension protocol was generally associated with those samples with higher percent fines and organic matter (**Figure 5**: *Scatterplot of % change in normal survivors vs % fines*, **Table 2**). Comparing the outcome of the resuspension protocol to the PSEP protocol, the following were observed:

- 15 of 31 treatments were unchanged
- 8 of 16 SQS exceedances changed to passes
- 5 of 6 CSL exceedances changed to passes
- 1 of 6 CSL exceedances changed to an SQS exceedance
- 2 passes changed to SQS exceedances as a result of improved reference performance

The improved recovery of normal larvae was seen in 29 of 31 test sediments and in 4 of 6 reference sediments. This supports the conclusion that the resuspension protocol provides an improvement for the bivalve larval bioassay in sediments where entrapment occurs. This potential for entrapment can be partly determined by looking at the percent fines in a sediment, but other factors such as the presence and nature of wood waste should also be considered. It is interesting to note that improved recovery in fine-grained reference sediments reduces the frequency of reference failures and may result in some test sediments failing that would otherwise have passed using the standard PSEP protocol. This occurred for 2 of the 31 test sediments from the Port Gamble case study.

PROPOSED CLARIFICATIONS

The DMMP agencies and the Sediment Management Standards program propose the following change to the protocol for the *Neanthes* growth test:

1) Report results on both an ash-free dry-weight basis and on a dry-weight basis. The AFDW procedure eliminates weight from sediment in the gut, thereby providing a more accurate measurement of the change in biomass during the exposure period.

The DMMP agencies propose the following clarification regarding the sediment bivalve larval test:

2) Substitute the resuspension procedure for the standard PSEP protocol when using the bivalve larval bioassay to test sediments with high concentrations of fines, wood waste or other flocculent material. This decision should be made in coordination with the DMMP agencies. For routine testing of sediments with lower fractions of fines, wood waste or flocculent material, the standard PSEP protocol should be used. However, dredging project proponents may elect to use the resuspension protocol if they have concerns about false positives due to entrapment.

The Sediment Management Standards program proposes the following clarification to the sediment bivalve larval test:

3) Substitute the resuspension procedure for the standard PSEP protocol when using the bivalve larval test to determine compliance with the SMS for sediments with high concentrations of fines, wood waste or other flocculent material. For routine testing of sediments with lower fractions of fines, wood waste or other flocculent material, the standard PSEP protocol will be used. However, Ecology or the PLP may elect to use the resuspension protocol at their discretion if they have concerns about false positives due to larval entrapment.

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Table 1. Bioassay Protocol Refinements History in the Dredged Material Management Program (DMMP) and Sediment Management Standards (SMS) Program

Year	Title	Paper Type
2008	Reference Areas for Freshwater Bioassays	Clarification
2005	**Sediment Larval Test Species Recommended for Toxicity Testing by the DMMP Program**	Clarification
2004	**Ammonia and Sulfide Guidance Relative to *Neanthes* Growth Bioassay**	Clarification
2002	Ammonia and Amphipod Toxicity Testing	Clarification
2001	**Reporting Ammonia LC50 Data for Larval and Amphipod Bioassays**	Clarification
1998	BIOSTAT Software for the Analysis Of DMMP/SMS	Technical
1999	Use Of Amphipod, *Eohaustorius Estuarius,* Relative to Grain Size and Salinity	Clarification
1997	Selection of Negative Control Sediments and Use of Control Sediments as Reference Sediments	Clarification
1996	Statistical Evaluation of Bioassay Results	Clarification
1996	**Neanthes 20-Day Bioassay - Further Clarification on Negative Control Growth Standard, Initial Size, and Feeding Protocol**	Clarification
1995	**Interim Growth Rate and Mortality Guidelines for the *Neanthes* 20-Day Growth Bioassay**	Clarification
1995	In-Batch Testing for Reference Sediments for PSDDA Bioassays	Clarification
1994	Restriction on exotic species importation	Clarification
1994	**Interim Revised Performance Standards for the Sediment Larval Bioassay**	Revised Clarification
1994	***Neanthes* 20-Day Bioassay - Interpretation Clarifications**	Clarification
1993	Species Substitution for the 10-Day Amphipod Bioassay	Clarification
1993	**The *Neanthes* 20-Day Bioassay - Requirements for Ammonia/Sulfides Monitoring and Initial Weight**	Clarification
1992	**Implementation of the *Neanthes* 20-Day Sediment Bioassay**	Issue
1991	Modifications to Holding Time for Biological Testing	Issue
1991	Echinoderm Embryo Sediment Bioassay Protocol	Clarification
1991	PSDDA Requirement to Collect and Report Amphipod Reburial Data	Clarification
1990	Wet Sieving Method for Reference Sediment Grain Size Matching	Clarification
1990	Requirements for Analyzing Sediment Conventionals	Clarification
1990	Echinoderm Bioassay Guidelines	n/a
1990	Collection of Reference Sediment Samples	n/a
1990	Amphipod Bioassay Protocol	n/a
1990	Activities to Provide Better Reference Areas	Status

Documentation for changes on DMMO website at:
http://www.nws.usace.army.mil/PublicMenu/Menu.cfm?sitename=dmmo&pagename=Bioassays

Table 2. Bivalve Larval Test, Port Gamble Remedial Investigation 2011. Case study comparing of outcomes based on PSEP and Resuspension protocols.

Treatments	Percent Fines	Mean Number Normal PSEP*	Mean Number Normal Resuspension*	Signficance Relative to Reference	Standard	Resuspension
PG11-BW-01-S	18.10	81.4	84.3	N**	Pass	Pass
PG11-BW-02-S	8.80	91.3	81.5	N → S	Pass	Pass
PG11-BW-03-S	21.10	89.4	78.3	N → S	Pass	Pass
PG11-BW-04-S	71.00	60.7	84.5	S → N	CSL	Pass
PG11-BW-05-S	64.30	62.6	88.7	S → N	SQS	Pass
PG11-BW-06-S	66.20	52.8	90.4	S → N	CSL	Pass
PG11-BW-07-S	53.80	68.0	87.3	S → N	SQS	Pass
PG11-BW-08-S	88.20	70.4	84.8	S → N	SQS	Pass
PG11-BW-09-S	86.40	63.5	83.1	S → N	SQS	Pass
PG11-BW-10-S	81.20	54.8	86.8	S → N	CSL	Pass
PG11-BW-11-S	85.70	61.6	69.0	S	SQS	SQS
PG11-BW-12-S	48.40	64.9	65.2	S	SQS	SQS
PG11-BW-13-S	87.20	56.1	72.3	S	CSL	Pass
PG11-BW-14-S	90.00	70.0	79.6	S → N	SQS	Pass
PG11-BW-15-S	90.10	63.0	69.7	S	SQS	SQS
PG11-BW-16-S	92.90	66.5	63.2	S	SQS	SQS
PG11-BW-17-S	30.80	70.9	73.7	S	SQS	Pass
PG11-BW-18-S	86.40	81.4	84.3	S	CSL	SQS
PG11-BW-19-S	95.30	91.3	81.5	S	SQS	SQS
PG11-BW-20-S	96.50	58.7	75.3	S	SQS	Pass
PG11-BW-21-S	95.30	51.0	72.8	S	CSL	Pass
PG11-MS-01-S	27.40	72.9	96.6	N	Pass	Pass
PG11-MS-02-S	18.00	73.3	97.8	N	Pass	Pass
PG11-MS-03-S	25.50	70.9	88.9	S	Pass	Pass
PG11-MS-04-S	55.80	66.7	92.6	S → N	SQS	Pass
PG11-MS-05-S	17.10	75.3	84.0	S	Pass	Pass
PG11-MS-06-S	50.80	65.1	77.4	S	SQS	SQS
PG11-MS-07-S	32.70	62.3	75.5	S	SQS	SQS
PG11-MS-08-S	7.10	76.3	80.2	S → N	Pass	SQS
PG11-MS-09-S	16.30	63.9	71.6	S	SQS	SQS
PG11-MS-10-S	38..5	67.5	80.4	S	Pass	SQS

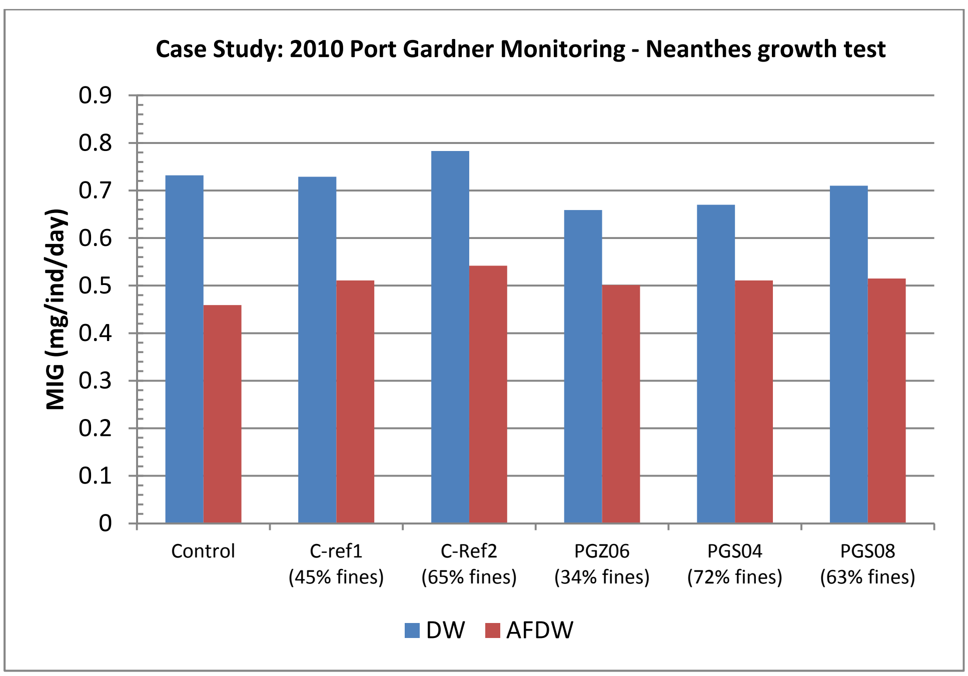
* Mean Number Normal = Normal Test Sediment/Normal Reference Sediment

**N=Not Significant, S= Significant

Figures 1-5 (Case Studies: DMMP and SMS).

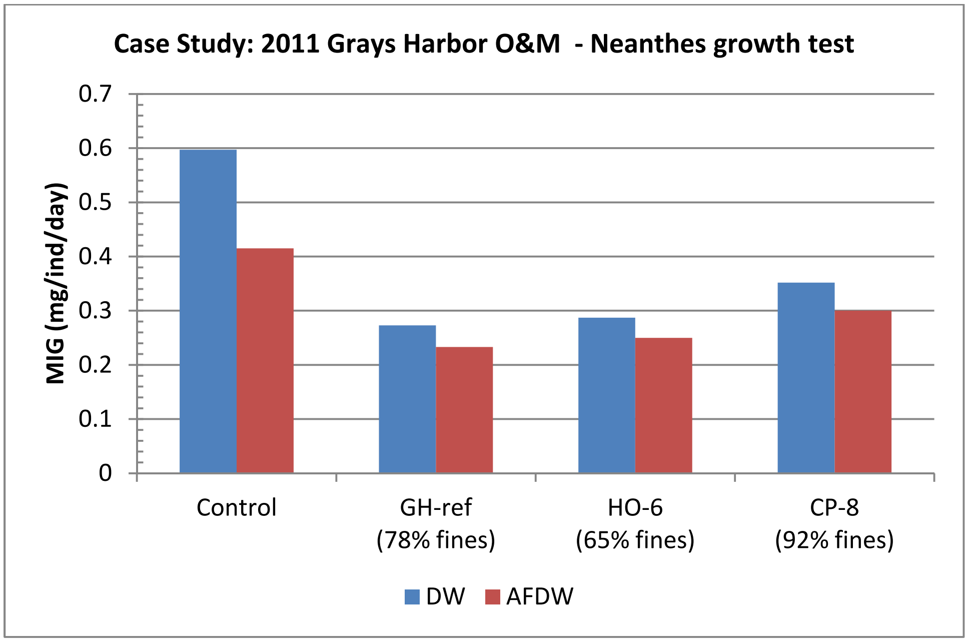
A) Case Studies: *Neanthes* growth test[1]:

Change in MIG (Mean Individual Growth) based on the three measures relative to control, reference and treatments. Biggest impact has been observed in control versus reference performance evaluations for Grays Harbor O&M Characterizations in 2011 and 2012, where the reference failed the control performance guidelines as illustrated in Figures 1b-c.

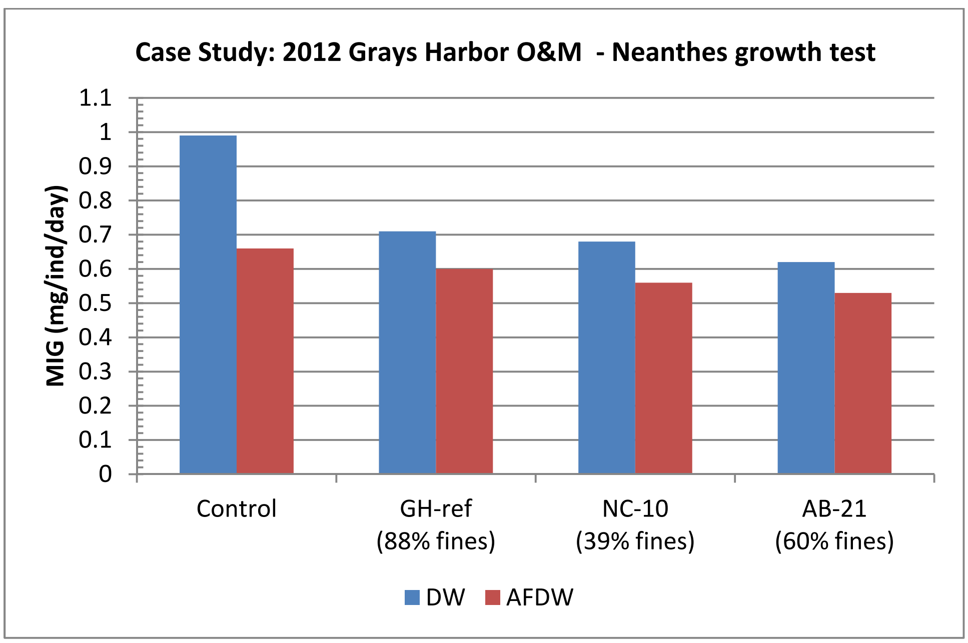


1a) Port Gardner Disposal site monitoring – 2010 (No hits)

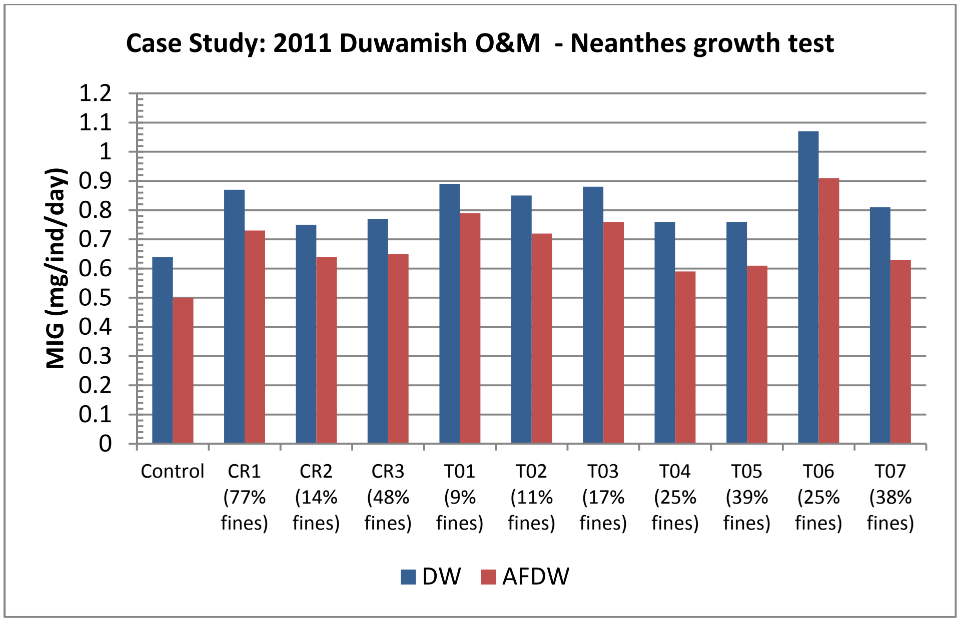
[1] Percent fines denoted in parenthesis on all figures

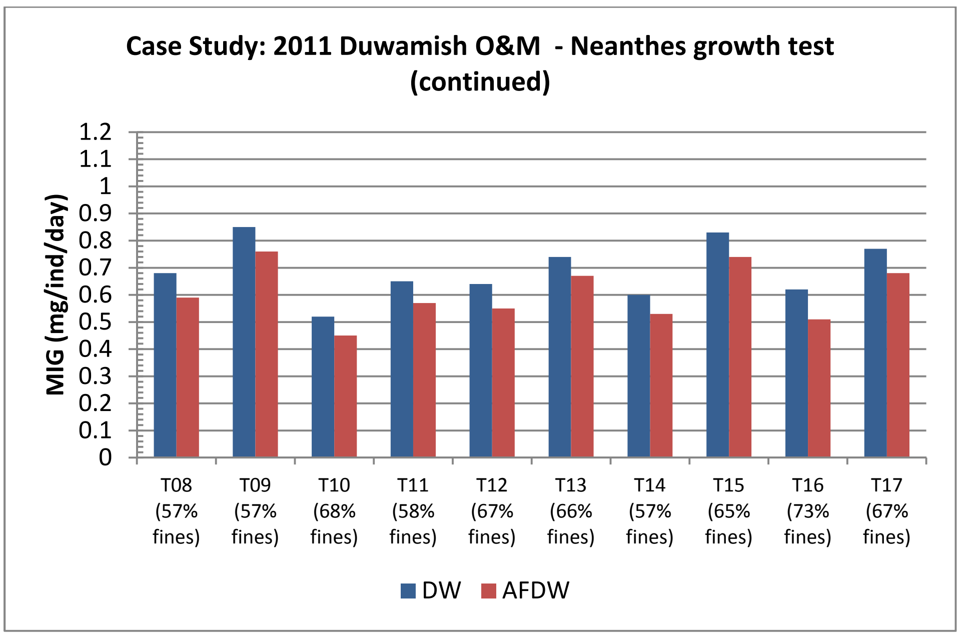


1b) Grays Harbor O&M Characterization – 2011 (No hits)



1c) Grays Harbor O&M Characterization – 2012 (No hits)





1d) Duwamish O&M Characterization – 2011 (17 DMMUs characterized) (No hits)

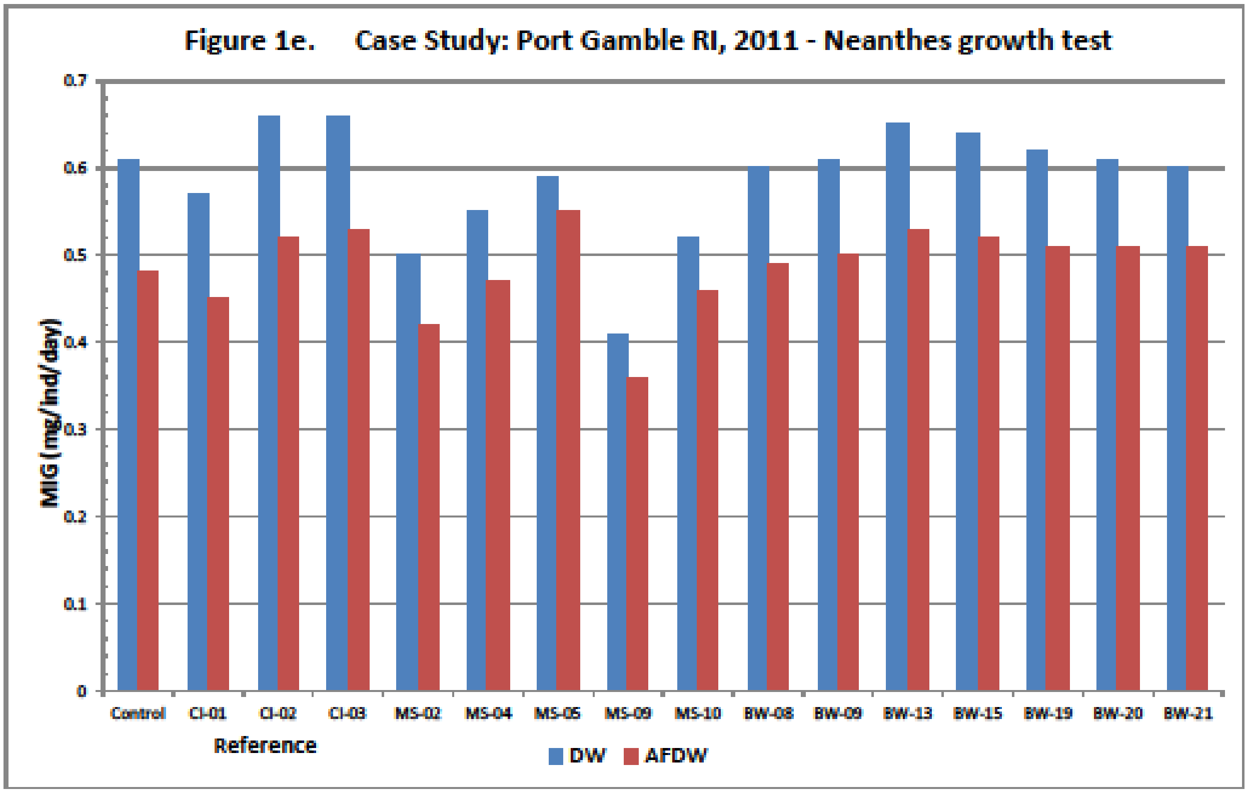


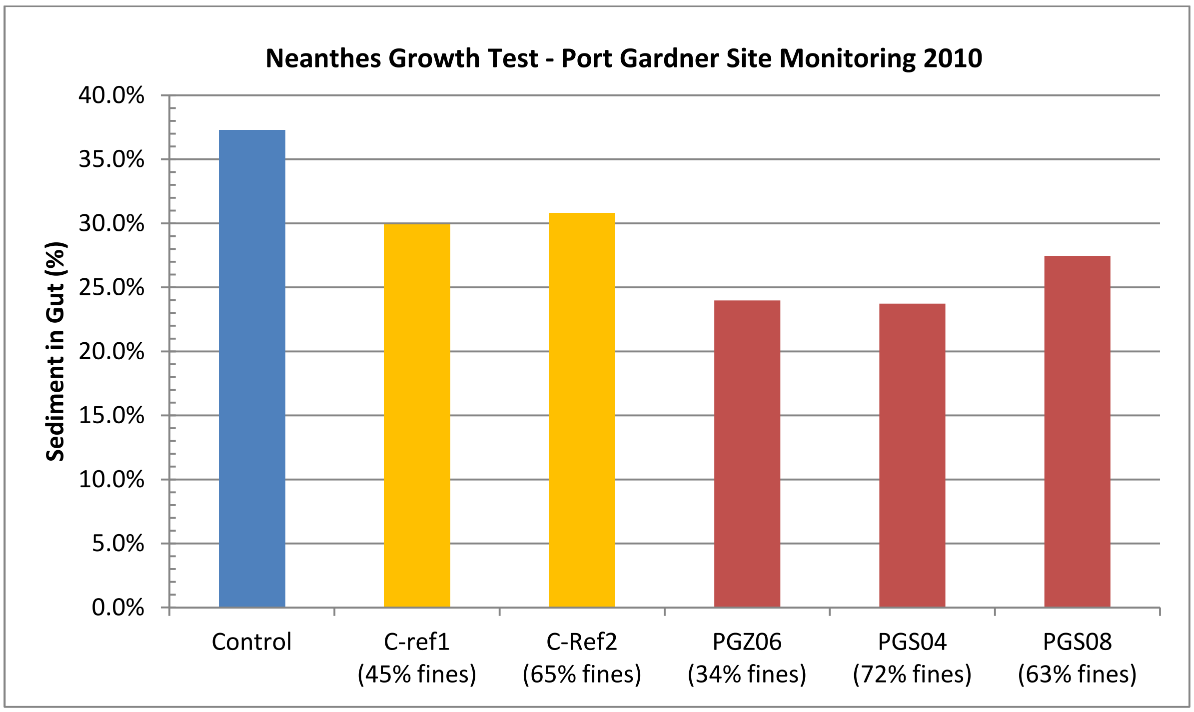
Figure 1e. SMS Case Study: Port Gamble RI, 2011 (No SQS Hits).

Percentage of organism dry weight that is attributable to sediment retained in the gut:

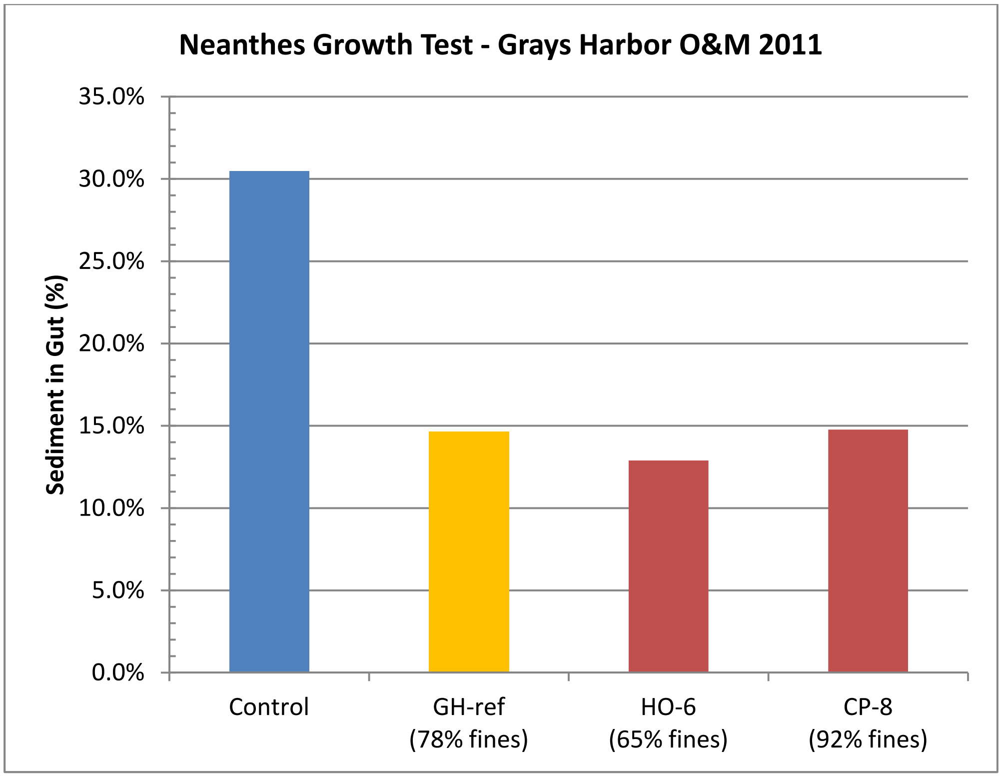
Controls: DMMP Average: 31 % (22 – 37%); SMS Average: 21%

Reference: DMMP Average: 20 % (15 – 31%); SMS Average: 21%

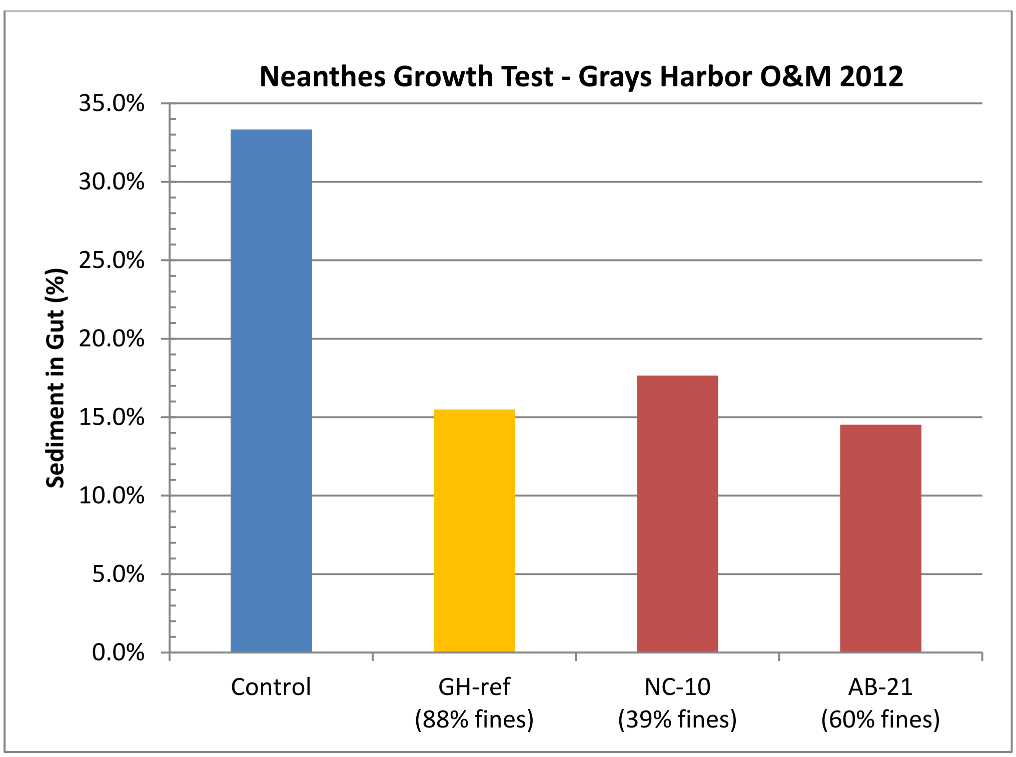
Treatments: DMMP Average: 18% (11 – 28%); SMS Average: 15 % (7 – 18%)



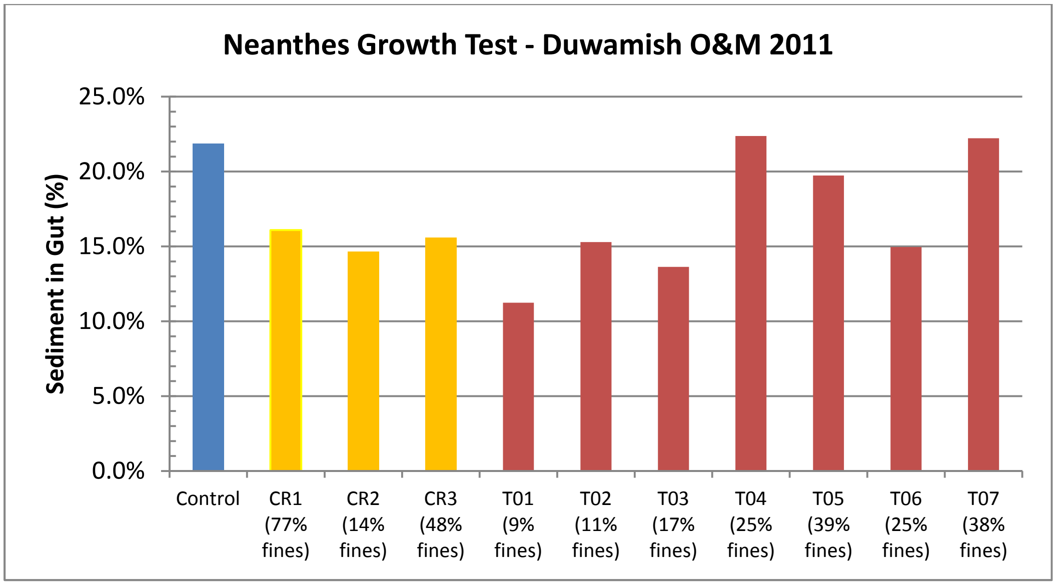
2a) Port Gardner Disposal Site Monitoring 2010 – comparative sediment in gut

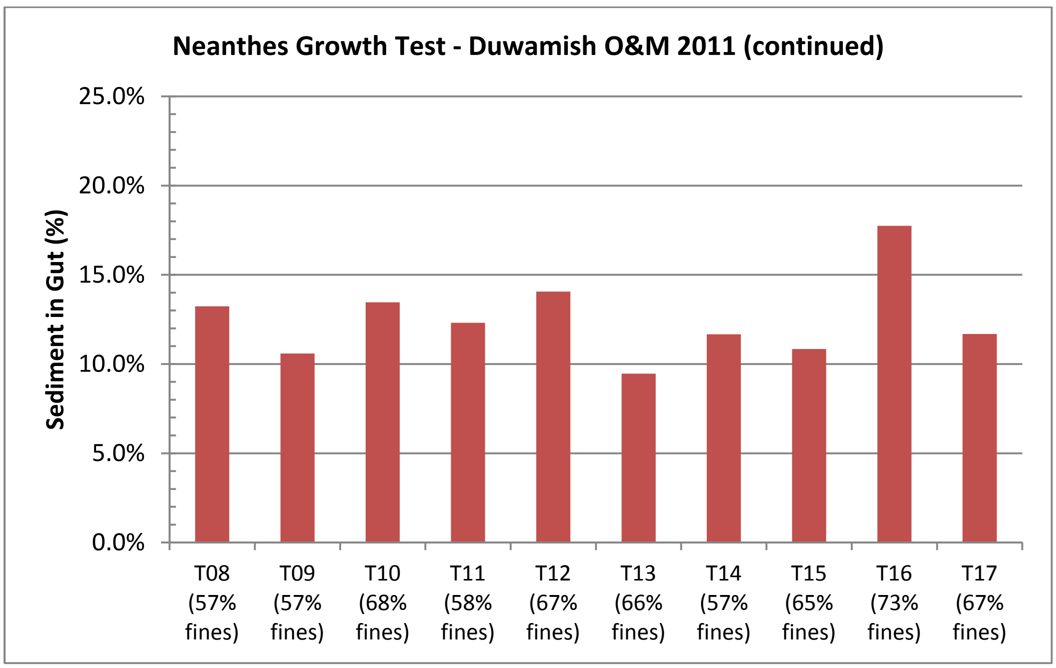


2b) Grays Harbor O&M 2011 – comparative sediment in gut

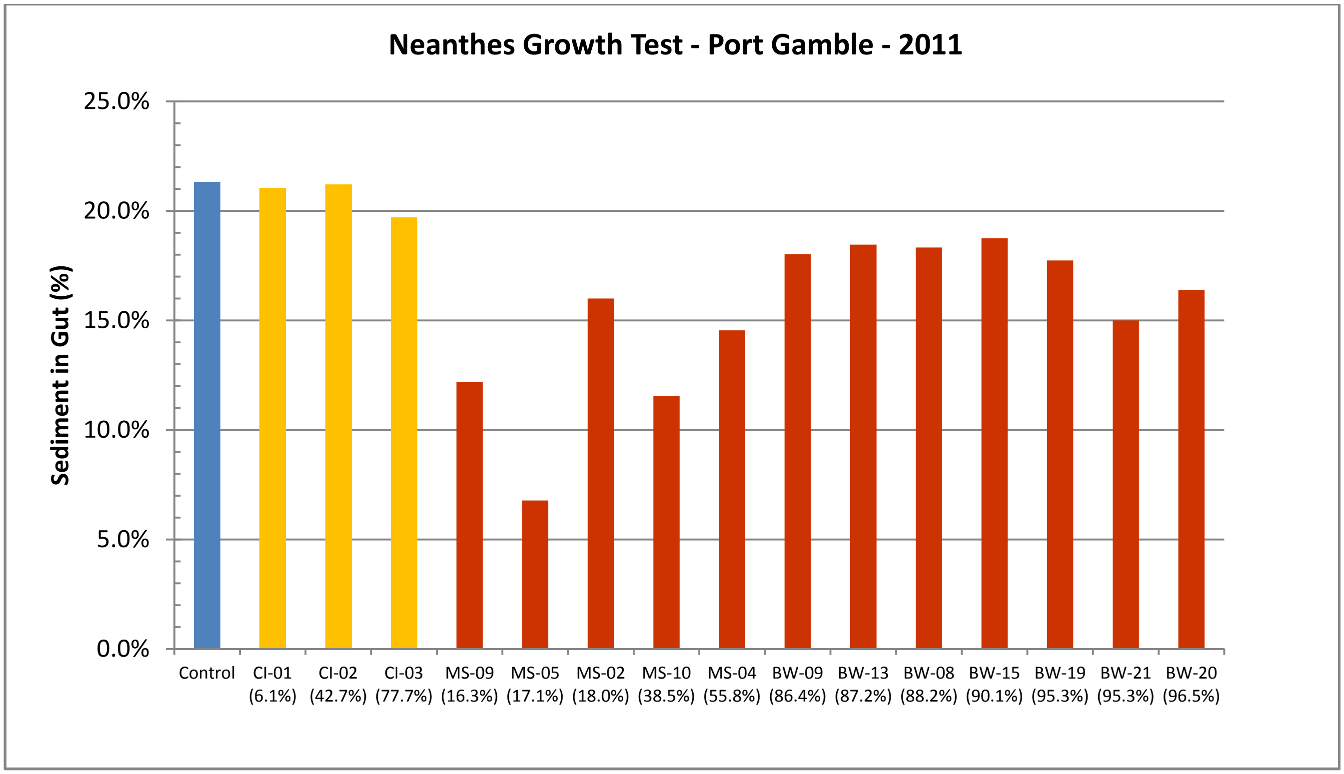


2c) Grays Harbor O&M 2012 – comparative sediment in gut



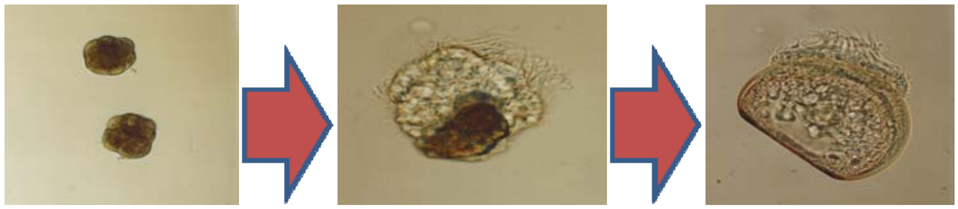


2d) Duwamish O&M 2011 – comparative sediment in gut (17 DMMUs total)



2e) SMS-Port Gamble, RI – 2011 – comparative sediment in gut

B) DMMP / SMS Case Studies: Bivalve Larval Test (*Mytilus galloprovincialis*):



Early in test
Non-swimming larvae rest on bottom, early cell division

Approximately 12-24 hours
Swimming forms rise off of sediment, begin gut/shell development

Later in test
Swimming, normal D-larvae, feeding in water column

Comparative differences between PSEP protocol versus Resuspension protocol:

PSEP Control DMMP Average: 91.4 % (84.8 – 94.4%)

Resuspension DMMP Control Average: 95.2 % (86 – 99.7%)

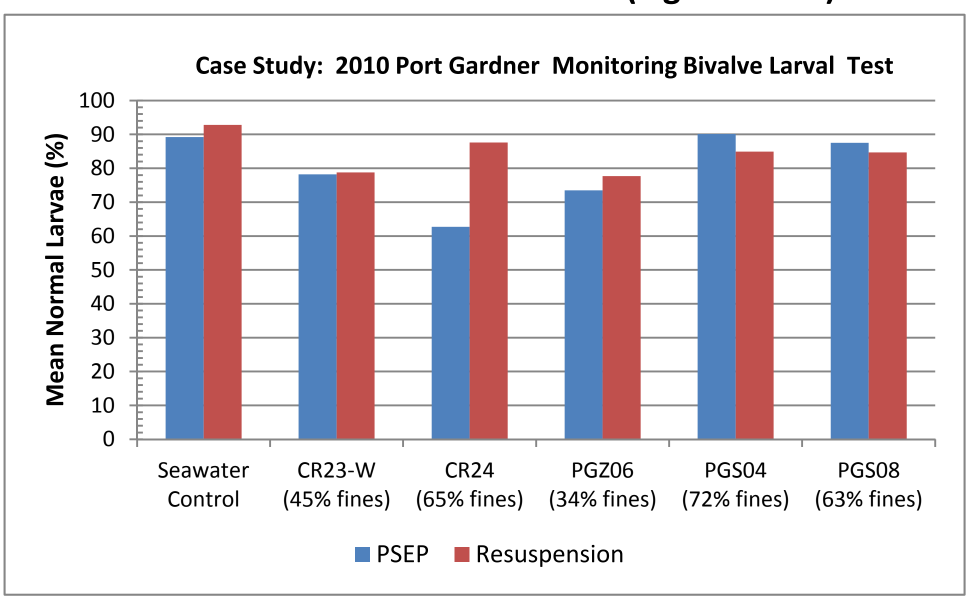
PSEP DMMP Reference Average: 82.6 % (62.7 – 92.4%)

Resuspension DMMP Reference Average: 83.0 % (78.8 – 87.6%)

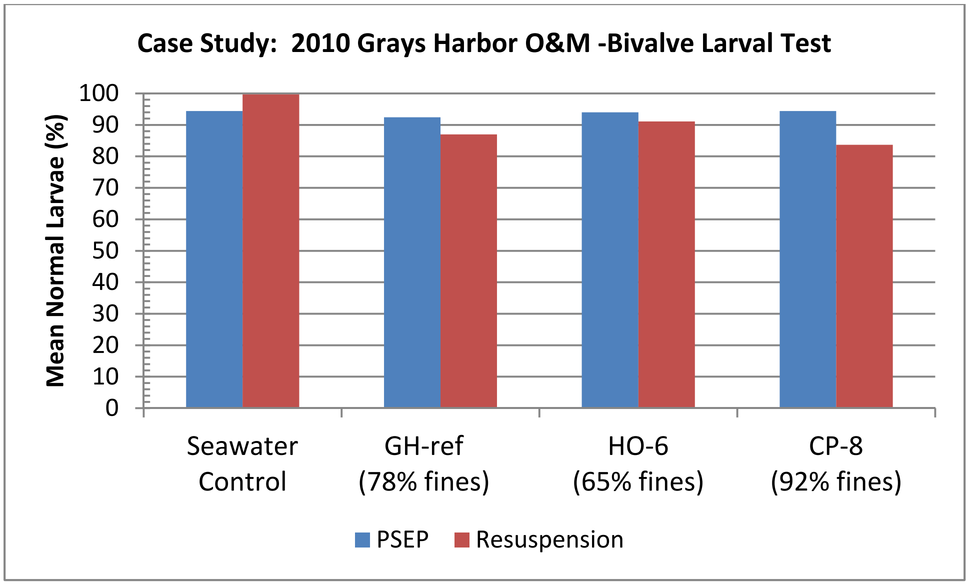
PSEP DMMP Treatment Average: 80.0 % (43 – 94.4%)

Resuspension DMMP Treatment Average: 77.1 % (30.9 – 98.6%)

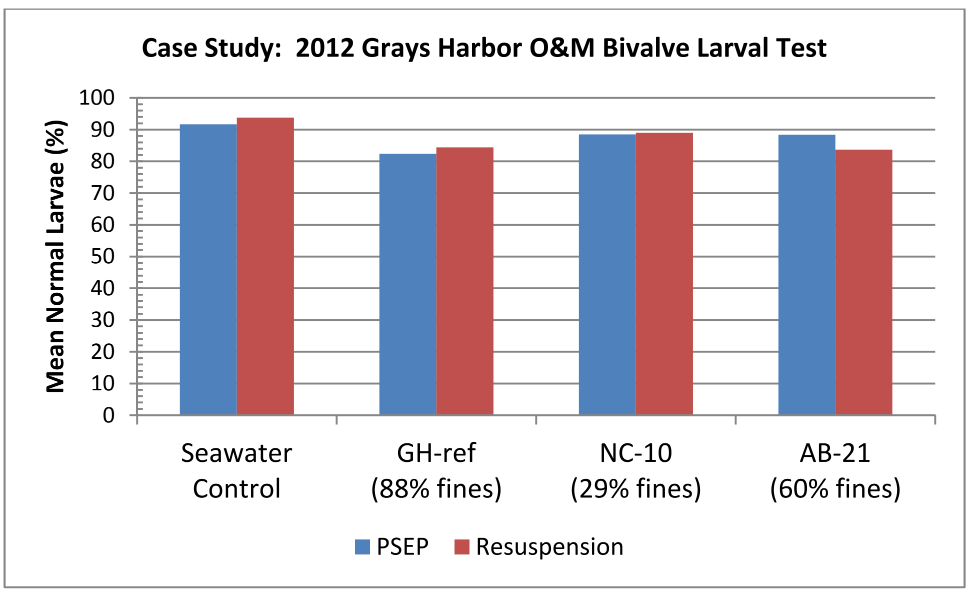
DMMP Case Studies: Bivalve Larval Test (Figures 3a-d):



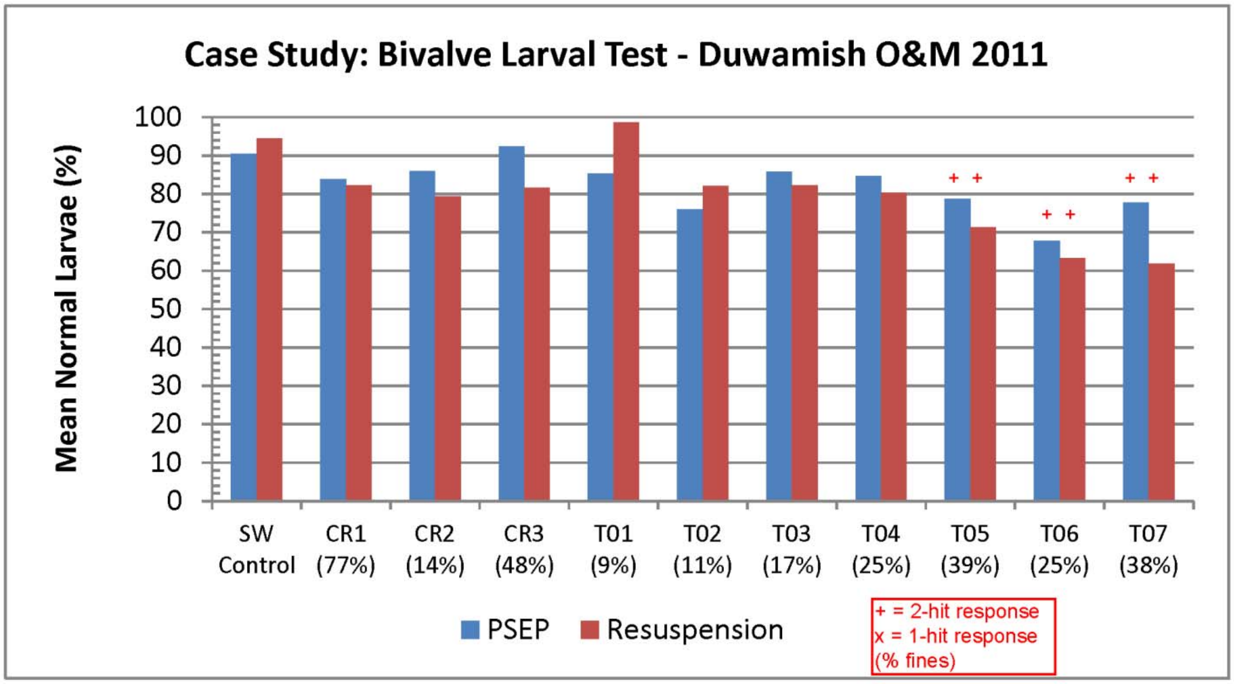
3a) **Port Gardner Disposal Site Monitoring – 2010 (No hits)**

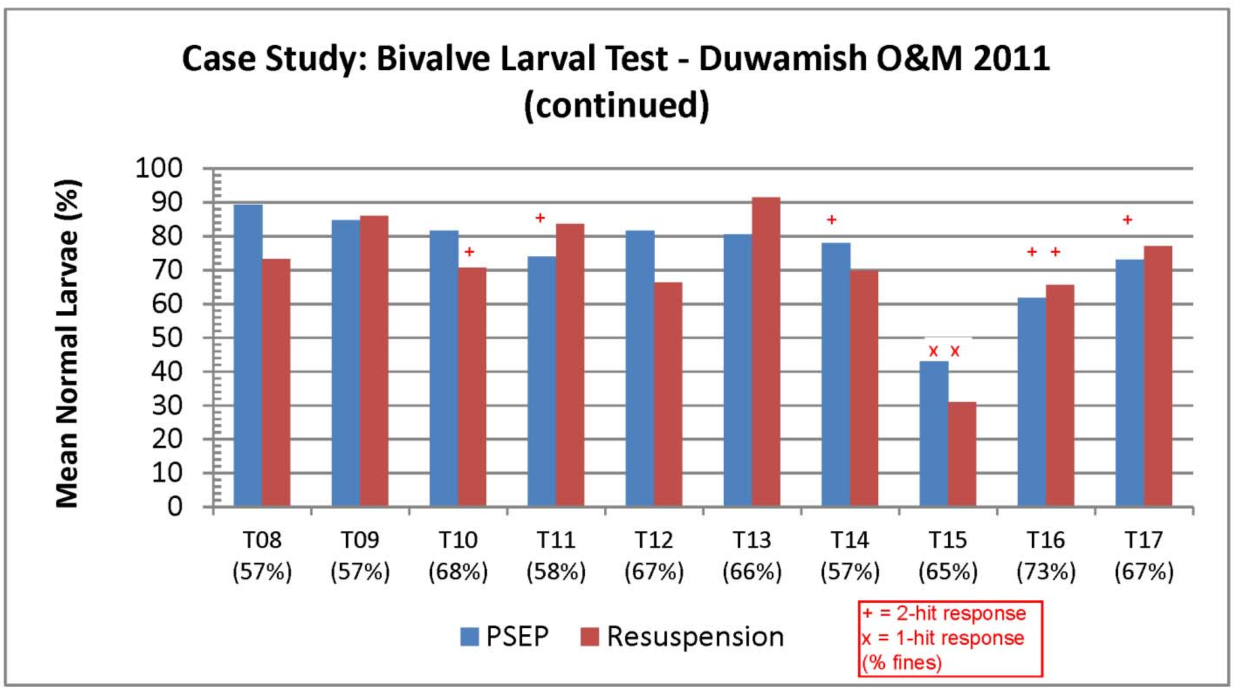


3b) Grays Harbor O&M Characterization – 2011 (No hits)

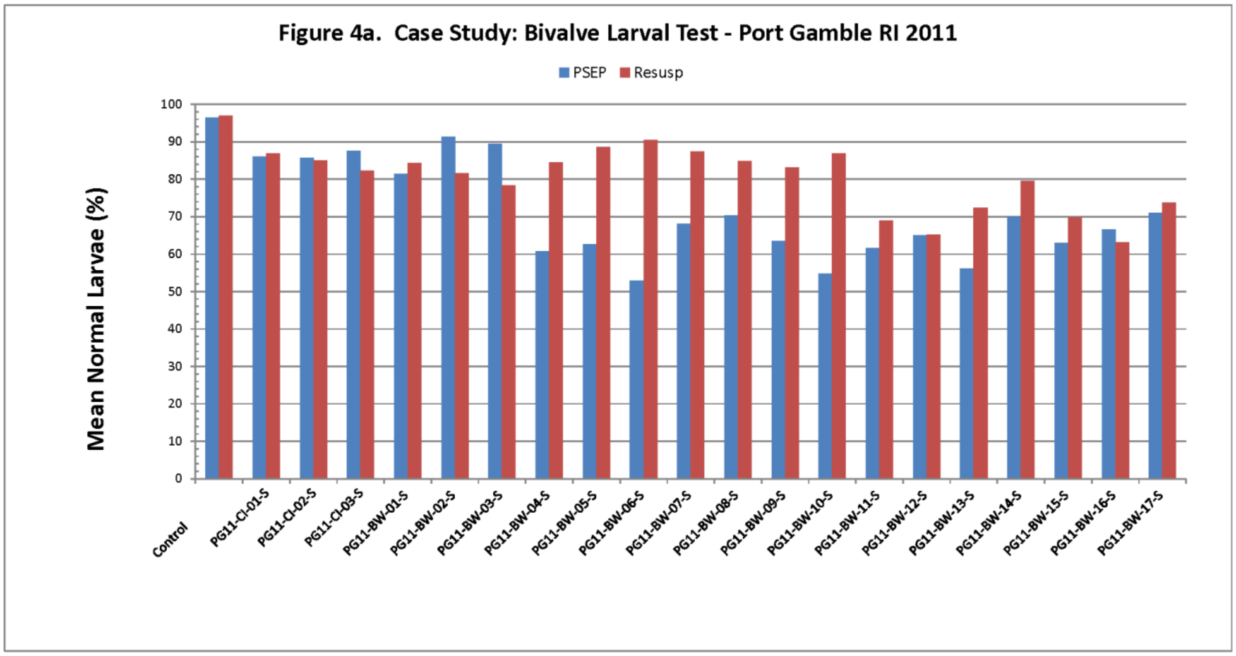


3c) Grays Harbor O&M Characterization – 2012 (No hits)





3d) Duwamish O&M Characterization – 2011 (2-hit responses observed for either PSEP and resuspension protocol did not change the overall bioassay interpretation relative to DMMP guidelines, as there were no other corroborating hits from other two bioassays (Amphipod and Neanthes). Only DMMU-15 failed based on 1-hit responses from both protocols.)



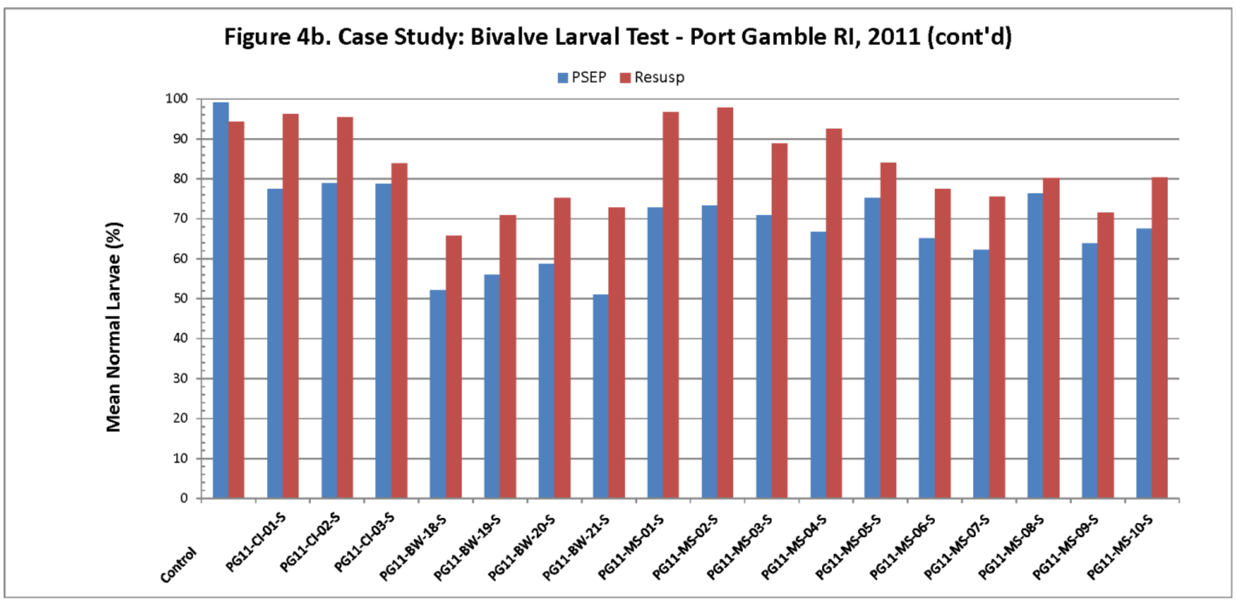
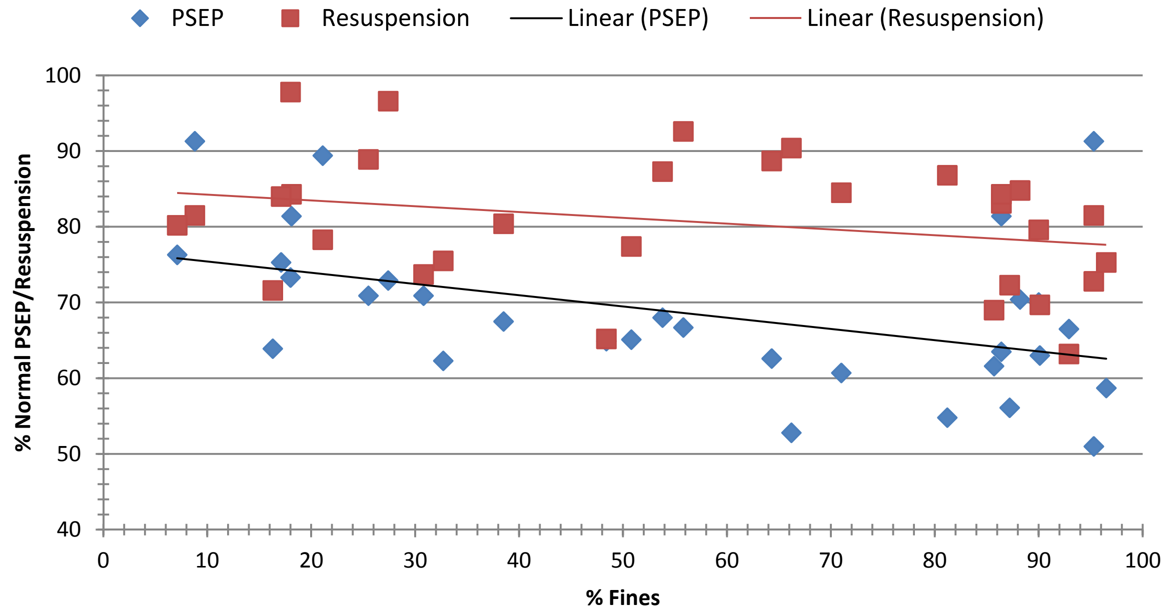


Figure 4a-b. SMS Case Study Port Gamble Remedial Investigation Study, 2011, comparing outcomes from PSEP and Resuspension protocols (See Table 2 for interpretation outcomes for testing results).

Figure 5. Scatterplot of % Fines versus PSEP and Resuspension Protocol (Port Gamble Remedial Investigation 2011), Bivalve Larval Bioassay



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Attachment 1 (Revised: 4/1/13).

A) *Neanthes* Growth Bioassay – Ash-Free Dry Weight (AFDW) Protocol

Ash-free dry weight (AFDW) represents the mass of biological tissues without the weight of inorganic materials (e.g. sediment) in the gut of worms. In order to determine the AFDW for the 20-day Neanthes growth bioassay, the following procedures should be followed:

1. Terminate each test chamber as indicated in the standard PSEP method;
2. Rinse sediment off of test organisms and place all worms from each test chamber into a labeled, pre-ashed (550^0C for 2 hours), and tared aluminum weigh boat (note that weigh boat should be labeled in a manner that will not be removed in the ashing process, e.g. etching);
3. Dry tissues for 24 hours at 60^0C;
4. Measure and record "dry weight";
5. Bake tissues in Muffle Furnace at 550^0C for 2 hours to remove all tissues;
6. Using forceps, transfer weigh boats to a dessicator to allow contents to cool;
7. Using a microbalance, measure and record the "Ashed Weight" (Weight of all inorganic material);
8. Subtract the ashed weight from dry weight to determine the ash-free dry weight; and,
9. Both dry weight and AFDW should be reported.

B) Bivalve-Larval Resuspension Termination Protocol-

The purpose of the larval resuspension method is to account for any larvae that may be buried in significant layers of sediment on the bottom. In reference or test sediments, the exclusion of larvae may result in reference failure or false positives.

The resuspension test is initiated and conducted in a manner similar to the standard PSEP method. As with the standard PSEP method, the test is terminated when approximately 95 percent of the embryos in the seawater control have reached the prodissoconch I stage, 48 to 60 hours after test initiation. For the resuspension termination protocol, the contents of the test chamber are gently resuspended using a perforated plunger at approximately 42 hours from test initiation. The test is then terminated in a manner consistent with the standard method, when 95% of the control larvae have achieved the prodissoconch I stage.

The bioassay is terminated in the following manner.

1. The bivalve larval resuspension test is terminated when development in the seawater controls is approximately 95% prodissoconch I;
2. At approximately 42 hours from test initiation, gently resuspend the contents of each test chamber using a perforated plunger. Mix for approximately 10 seconds or until the water, larvae, and settled sediment are resuspended in each container. Care should be taken to rinse the plunger between test chambers to prevent the transfer of larvae. Note the time that resuspension was initiated for the test.
3. Control and reference treatments should also be mixed at this time.
4. The test is terminated following the protocol presented in the standard PSEP method:
   a. The test is terminated at 48 to 60 hours, when approximately 95% of the surviving larvae in the controls have developed into D-shaped, prodissoconch I larvae.
   b. carefully pour the water overlying the sediment into a clean 1-liter beaker;
   c. mix the decanted water with a perforated plunger;
   d. collect 10-mL aliquots of the well-mixed sample by calibrated pipette and place in 20-25 ml sealable shell or scintillation vials; and,
   e. The contents of each vial are preserved with 0.5- 1mL of 5-percent buffered formalin or equilvalent.