

**Appendix 1**  
**Protocols for Implementing the**  
**Scope of Services to Address Aquatic Environment Issues,**  
**North Totten Inlet Site Proposed Mussel Culture**

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**Protocols for implementing the scope of services  
to address aquatic environment issues at the proposed North Totten Inlet mussel farm**

**1. Background.** These protocols implement the scope of work proposed in a letter from Aquatic Environmental Sciences to Taylor Resources, Inc. dated August 29, 2001. These studies address concerns raised by Thurston County with regard to the Taylor Resources proposal for a mussel culture operation in Totten Inlet, Washington. The results of these studies will be included in an Environmental Impact Statement being prepared in accordance with the Washington State Environmental Policy Act (SEPA).

Taylor Resources submitted an application for a Shoreline Substantial Development Permit (SSDP 961372) to expand mussel culture operations in Totten Inlet on November 13, 1996. Thurston County issued a SEPA Determination of Significance (SEPA File No. 961372) on September 9, 1998. Mr. James Driscoll, Thurston County Hearing Examiner, affirmed Thurston County's Determination of Significance (DS) on June 18, 1999 following an appeal by Taylor Resources. The DS listed the following possible significant adverse effects from expanded mussel culture operations in Totten Inlet:

- A. Impacts to the bottom dwelling organisms (benthic community).**
- B. Impacts to the surrounding water column.**
- C. Impacts to the phytoplankton resource and the effects that could have on other aquaculture and aquatic life.**
- D. Impacts caused by escapement and propagation of mussels.**
- E. Impacts caused by navigational lighting.**

Aquatic Environmental Sciences is providing services to Taylor Resources to address Items A and D, the benthic habitat and genetic elements. Item E, navigational lighting, will be addressed by others. The following protocols are based on well-focused questions that address the specific issues raised by Thurston County.

*A Literature Review Describing the Environmental Effects Associated with the Intensive Culture of Mussels* (copy attached) has previously been provided to improve the application of available scientific information to Totten Inlet. Combined with the results of site-specific field studies described below, the literature review will be used to confirm the parameters of the aquatic environment at the North Totten site, and to characterize potential impacts of an expanded mussel culture operation in the proposed location. The literature review, and the proposed *Scope of Work* upon which these protocols are based, have been submitted to Thurston County and their independent technical reviewers. If necessary, recommendations from the independent technical reviewers will be incorporated at a later date.

**2.0. Study design.** This study will rely on a regression approach for several reasons:

➤ The regression approach provides a continuum of information, allowing us to more efficiently model farm affects and to understand subtle physicochemical and biological responses to mussel farming. The purpose of this study is not to evaluate the statistical significance of differences in endpoints between a reference station and treatment stations. The purpose is to evaluate trends in these endpoints as a function of distance from the farm and to determine thresholds for biological effects by evaluating the covariance of biological and physicochemical endpoints. Three samples will be collected at a local reference station for comparison with

treatment stations. If one assumes equal variances for appropriate endpoints at treatment and reference stations (i.e. that  $s^2 = \sigma^2$  the population variance) then *t-tests* are possible assuming the mean at the reference station equals  $\mu$  (the population mean).

➤ The regression approach allows the use of other multifactor analytical techniques such as principle components analysis and factor analysis to better understand the inter-relationships between variables in complex datasets.

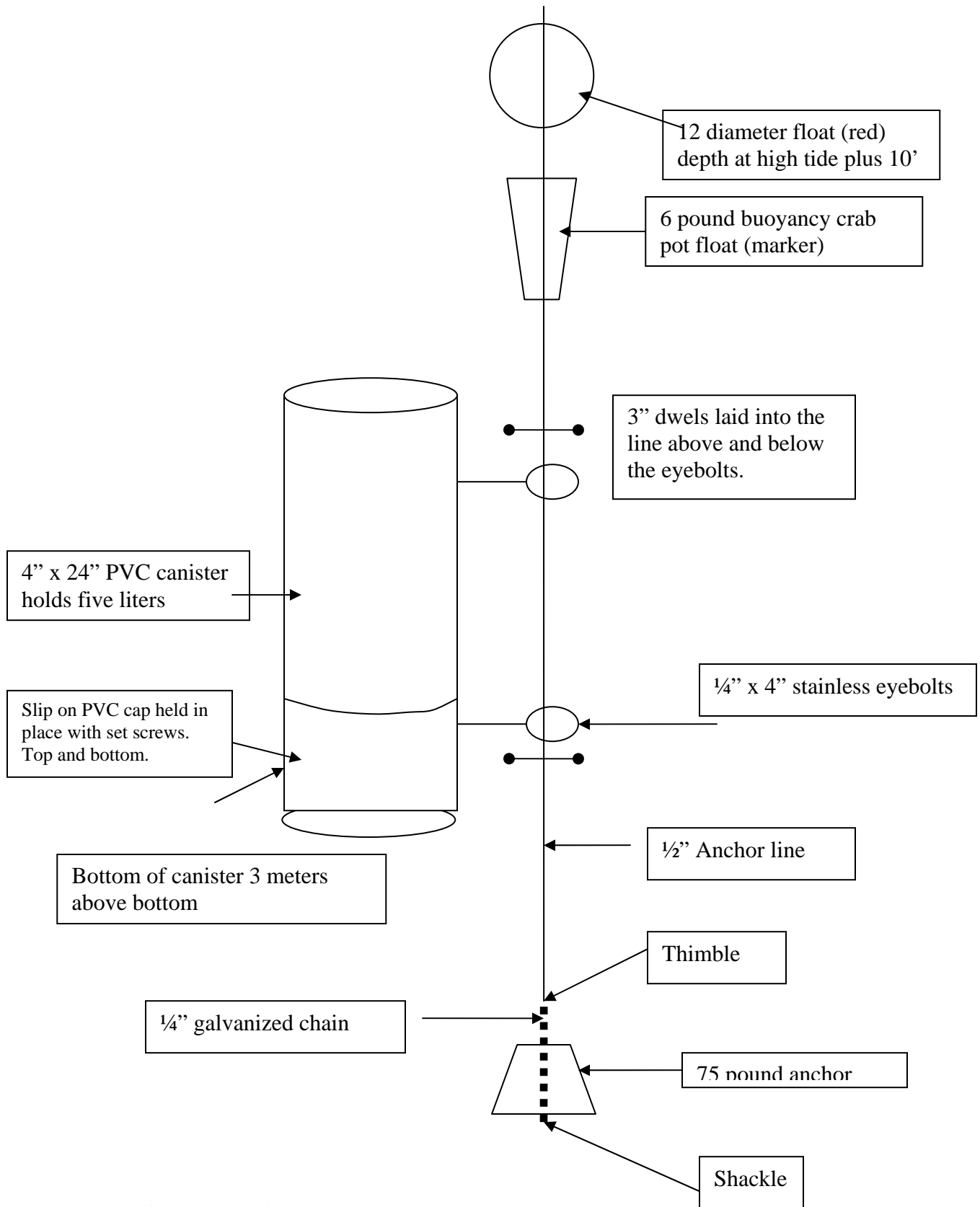
➤ The regression approach also allows for a better understanding of the temporal relationships between mussel consumption of seston, farm biodeposits, sediment physicochemistry, to include the recycling of nutrients, and the response of infauna and epifauna in the fouling community.

**3.0. Task A. Impacts to the benthic community.** The deep water and currents at the North Totten site will disperse feces and pseudofeces from mussels and their fouling biomass. Taylor Resources' mussel farm at Deepwater Point is similar to the proposed mussel culture operation at the North Totten Inlet site and will be used to evaluate the environmental response to mussel farming in Totten Inlet.

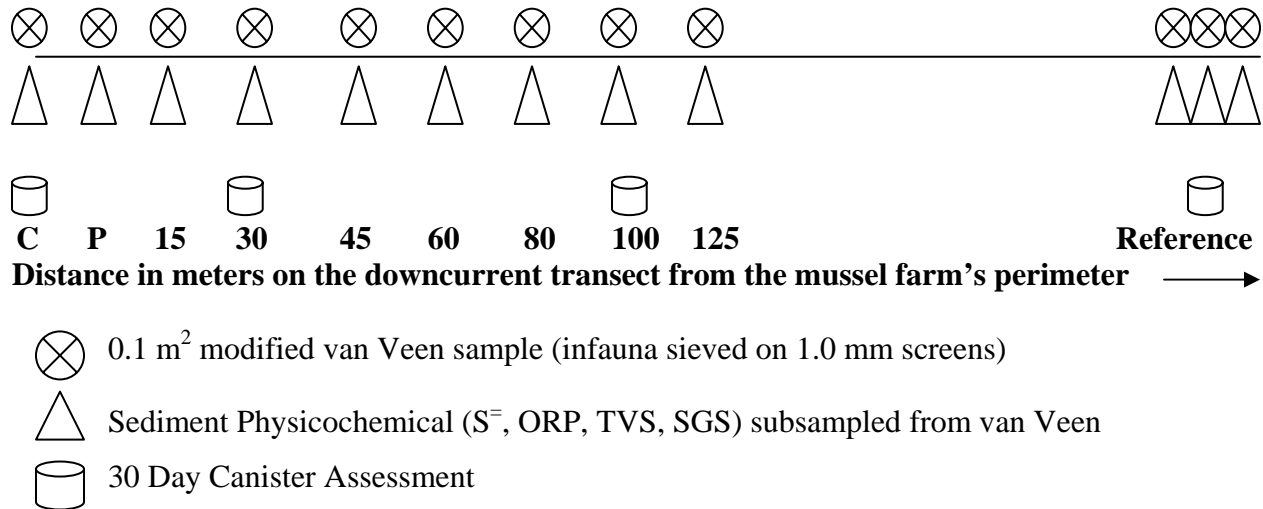
**3.1. Canister studies.** The rate of biodeposition from mussel cultures can be measured in a straightforward manner using sediment canisters at Deep Water Point. This element of the study design will provide comparisons of the particulate organic and inorganic matter collected in four 10 cm diameter canisters deployed 5 meters off bottom for 30 days bimonthly during the period September 2001 through July 2002. The canister design is described in Figure (1) and the location of each of the seven canisters along the sampling transect is identified in Figure (2).

The canisters will be anchored to the bottom using a suitable weight of at least 75 pounds. The contents of each canister will be fixed *in-situ* using 50 ml of formalin mixed with 500 ml 60 o/oo seawater (60 grams of NaCl in one liter of water). The 4-inch diameter canisters will be capped when brought onboard the recovery vessel and gravity filtered as soon as possible across a 50 micron Nitex filter. The solids retained on the filter will be backwashed with distilled water into pre-labeled one-liter HDPE bottles and fixed with 10 percent formalin. This residue will be dried at  $103 \pm 2$  °C and weighed to the nearest 0.1 mg in aluminum boats. The residue will then be ignited at  $550 \pm 10$  °C for one hour or until there is a less than 4% weight loss following additional combustion. Total Solids and Total Volatile Solids are calculated from the data obtained. Internal replicates are not possible. The canisters data is used to quantify the flux of biodeposits from the mussel farm to the benthos. These biodeposits result in changes in sediment physicochemistry leading to a biological response. These relationships are depicted diagrammatically below.

Farm biodeposits → Canisters → Sediment Physicochemistry → Biological change



**Figure 1. Canister design.**



**Figure 2. Sample station location and sample types for Deepwater Point mussel farm benthic surveys.**

**3.2. Task A2. Determining the benthic physicochemical and biological response to an existing mussel farm at Deepwater Point.** Determining the assimilative capacity of the sediments is more difficult. Assimilative capacity depends on the resident invertebrate community, sediment grain size distribution, bottom current speeds and dissolved oxygen concentrations, water temperature, salinity, allochthonous input, etc. All of these inputs vary significantly with season. Mussels produce feces as a metabolic byproduct and pseudofeces when the concentration of particulate organic and/or inorganic material in the water exceeds their digestive capacity. Particulate inputs will be measured in the canister study. Their physicochemical and biological effects will be assessed by evaluating sediment total sulfides (S<sup>-</sup>), oxidation-reduction potential (ORP), total volatile solids (TVS) and sediment grain size (SGS). Sediment physicochemical and biological (infaunal) response will be evaluated in twelve 0.1m<sup>2</sup> van Veen grab samples whose location is defined in Figure (2). Sediment samples will be collected in November 2001. Specific protocols for these analyses are defined in the following paragraphs:

**3.2.1. Sample collection.** Samples will be collected using a 0.1 m<sup>2</sup> modified van Veen grab. Acceptable samples must meet the requirements of PSEP (1996):

- The sampler must be deployed at a maximum speed of 30 cm/sec;
- A minimum sediment penetration depth of 4 cm is required;
- The retrieved sampler must contain overlying water, with low turbidity, indicating minimal leakage and disturbance;
- The retained sediment surface should be relatively flat indicating minimal disturbance or winnowing.

Overlying water must be siphoned from the sampler through a 1.0 mm sieve and the top two centimeters of the sediment subsampled for physicochemical analyses. The sampling protocol will use approximately 5% of the surface area of the van Veen grab. All samples will

be treated identically and the subsampling for physicochemical analyses will not compromise the quantitative aspects of the invertebrate community analysis. Subsampling from the same grab is considered necessary because the composition of the infaunal community is very sensitive to sulfides and the distribution of sulfides and other physicochemical parameters is patchy (variable). Therefore, it is necessary to measure physicochemical variables in the same sample used for invertebrate analysis.

**3.2.2. Station positioning.** The survey vessel will be positioned using a ½” polypropylene transect line secured to the perimeter of the mussel farm and at the sampling station on the survey vessel. For purposes of this monitoring, no requirement to correct for hydrowire angle is imposed. The latitude and longitude of each sample will be determined using differential GPS with an accuracy of  $\pm 3.0$  meters.

**3.2.3. Sample evaluation.** The following observations will be noted on the field log sheet:

- Station location. Recorded using Differential GPS with an accuracy of  $\pm 3.0$  meters
- Water depth
- Gross characteristics of the surficial sediment to include:
  - Texture and color
  - Biological structures (shells, tubes, macrophytes)
  - Presence of debris (wood chips, wood fibers, trash, etc.)
  - Presence of bacterial mats, feces, pseudofeces, etc.
  - Odor (hydrocarbons or hydrogen sulfide)
  - The pH of the sediments will be measured
- Grab penetration depth will be recorded
- Comments related to sample quality such as leakage, winnowing or undue disturbance.

**3.2.4. Subsamples** shall be taken using a stainless steel spoon or proprietary sampler with shoulders that are two cm in height. The top two centimeters of the retrieved sediment should be placed in a stainless steel bowl and gently homogenized for approximately 10 seconds. The homogenate is then placed in 125 ml polyethylene urine specimen jars for Sediment Grain Size, Sulfide and Total Volatile Solids analysis. This procedure will sample approximately 5% of the surface area of the 0.1 m<sup>2</sup> van Veen grab. One sample is adequate for TVS, ORP, SGS and sulfide analyses. When sulfide analysis is not accomplished within a few hours – then samples should be fixed with 0.5 ml of two normal zinc acetate. Unrepresentative material may be removed in the field and noted on the field log sheet. Unrepresentative material includes large mollusk shells, large organisms, large woody debris and large pieces of macroalgae.

**3.2.5. Sample containers, labeling and handling.** Physicochemical samples should be stored on ice in coolers in the field. TVS samples should be frozen within 14 days. This allows a six-month holding time (PSEP, 1996). Samples for analysis of Sediment Grain Size should be maintained at 4 °C until analyzed within six months.

Infaunal samples will be held in five gallon plastic buckets until sieved and fixed in 15% buffered formalin in sea water on the same or the next day following collection. These samples should be transferred to 70% alcohol (ethanol or isopropyl) within four to five days of fixing.

The bodies and caps of all sample containers shall be labeled with coded tags. A third (loose) label will be added to each infaunal sample. This label will stay with the infaunal sample throughout processing and should be archived with the samples at the end of the study.

**3.2.6. Cleaning of equipment between samples.** All equipment (dredge, sampling fixtures, etc.) should be thoroughly rinsed with ambient seawater between grab deployments to remove sediment and organisms. No other special cleaning requirements are considered necessary for these analyses.

**3.2.7. Total Volatile Solids (TVS) analysis.** Approximately 35 grams of each sample is used for this analysis. Samples may be stored at 4° C for 14 days or frozen at -18° C for a maximum of six months. Standard Method 2540.E or EPA Method 160.4 is to be used for this analysis. Samples shall be dried at  $103 \pm 2^\circ$  C in aluminum boats that had been pre-cleaned by combusting at 550 °C for 30 minutes. Drying should be continued until no further weight reduction is observed. The samples are then combusted at 550 °C for 30 minutes or until no further weight loss is recorded. Total Volatile Solids are calculated as the percent difference between the dried and combusted weights. Quality assurance involves triplicate analyses on one of every 20 samples or on one sample per batch if fewer than 20 samples are analyzed. A maximum of 20 percent Relative Percent Difference (of the percent silt-clay fraction) is established as the Data Qualification Control Limit.

**3.2.8. Sediment Grain Size (SGS) analysis** will be accomplished at the beginning and end of each study. Approximately 50 grams of surficial sediment will be taken to a depth of 2.0 cm for sediment grain size analysis. The sediments are wet sieved on a 0.064 mm sieve. The fraction retained on the sieve is dried in an oven at 92 °C and processed using the dry sieve and pipette method of Plumb (1981). The sieves used for the analysis should have mesh openings of 2.0, 0.89, 0.25 and 0.064 mm. Particles passing the 0.064 mm sieve are analyzed by sinking rates in a column of water (pipette analysis). Hydrometer tests are not an acceptable substitute.

**3.2.9. Redox potential.** This analysis should only be conducted in the field using the following, or similar, procedures. Aquatic Environmental Sciences uses an Orion™ advanced portable ISE/pH/mV/ORP/temperature meter model 290A with a Model 9678BN Epoxy Sure-Flow Combination Redox/ORP probe. The meter's accuracy in the ORP mode is  $\pm 0.2$  mV or  $\pm 0.05\%$  of the reading, whichever is greater. Redox potentials evaluated in the laboratory following shipment were found to be unrepresentative.

**3.2.9.1. Calibration of the Redox Electrode:** Calibration reagents should be prepared 12 to 24 hours before use and held refrigerated. Redox Standard A (0.1 M potassium ferrocyanide and 0.05 M potassium ferricyanide) is prepared by weighing 4.22 g  $K_4Fe(CN)_6 \cdot 3H_2O$  and 1.65 g  $K_3Fe(CN)_6$  into a 100-ml volumetric flask. Approximately 50 ml of distilled water is added with swirling to dissolve the solids. The solution is then diluted to volume (100 ml) with distilled water. Standard B (0.01 M potassium ferrocyanide, 0.05 M potassium ferricyanide, and 0.36 M potassium fluoride) is prepared by weighing 0.42 g  $K_4Fe(CN)_6 \cdot 3H_2O$ , 1.65 g  $K_3Fe(CN)_6$ , and 3.39 g  $KF \cdot 2H_2O$  into a 100 ml volumetric flask. 50 ml of distilled water is added to dissolve the solids, and the solution is diluted to 100 ml with distilled water. A KCl filling solution for the internal reference electrode is prepared by placing 2.5 ml of 4 M KCl in 50 ml and diluting with distilled water to give a 0.2 M KCl solution.

Redox standards are used to calibrate electrodes at ambient temperature (15 to 20° C) at the start and end of measurements for each batch of samples. Standard A is transferred to a 150-ml beaker, and the electrode placed in the solution until the reading stabilizes with stirring (1 to 2 minutes). The potential of Standard A is approximately  $+147 \pm 9$  mV. The electrode is rinsed with distilled water and the measurement repeated with Standard B (potential of  $+216 \pm 9$  mV). The potential in Standard A is approximately + 69 mV greater than in Standard B.

The potential of the reference electrode (+244 mV at 20°C), corrected for the average difference between measured potentials of standard solutions and their calibration values, is added to the mV reading to determine the actual Eh potential in sediment samples. Eh potentials of approximately +300 to +350 mV are typical of oxygenated seawater. The redox electrode should be rinsed with distilled water after use and stored for short periods (a few weeks) in tap water. For longer periods, the electrode is drained, rinsed with distilled water, and stored dry.

**3.2.9.2. Measurement of sediment redox potential.** A 100 ml sample was collected for measurement of sediment redox and total sulfides. It is recommended that redox be measured before subsampling for total sulfide analysis. In the future these analyses will be accomplished in the grab prior to collecting subsamples. The Eh electrode was inserted into the sediment sample and a mV reading recorded after one to two minutes. The electrode was then removed, gently wiped free of sediment and used to measure the next sample. The probe was recalibrated at least once every two hours. Probes were rinsed in distilled water and stored in pH 7.0 buffer between batches of samples.

**3.2.9.3. Quality assurance procedures for the measurement of redox potential.** Triplicate analyses were conducted on one of every 20 samples, or on one sample per batch, if less than 20 samples are analyzed. No Data Qualification Control Limit is established for this test at this time.

**3.2.10. Total sulfide analysis.** Samples can be collected in glass or plastic containers. A minimum sample size of 50 grams is recommended. If unrepresentative material is to be removed from the sample, it should be removed in the field under the supervision of the chief scientist and noted on the field log sheet. Samples that will not be analyzed in the field within 24 hours should be preserved by adding 2 N zinc acetate solution (approximately 0.5 ml per 250 grams of sediment) and swirling the mixture. Samples should fill the sample container with no overlying air space. The caps should be tightly secured to prevent the entry of air.

Samples should be stored in the dark at 4° C and analyzed as soon as possible. It is important that air contact with the samples be minimized and that samples be kept moist to minimize oxidation. A maximum holding time of seven days is recommended for preserved samples.

**3.2.10.1. Calibration of the total sulfides field probe.** Because of the short (7 day) holding time and the remoteness of many salmon farms, it is recommended that this parameter be measured in the field within 24 hours. Aquatic Environmental Sciences uses an Orion™ advanced portable ISE/pH/mV/ORP/temperature meter model 290A with a Model 9616 BNC *Ionplus* Silver/Sulfide electrode. The meter has a concentration range of 0.000 to 19900 and a relative accuracy of  $\pm 0.5\%$  of the reading.

A sulfide antioxidant buffer solution (SAOB) is prepared in 250-ml HDPE screw-top jars by adding 20.00 g of NaOH and 17.90 g EDTA ( $\text{Na}_2\text{C}_{10}\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$ ) and diluting to 250 ml with



distilled water. The 2 M NaOH and 0.2 M Na<sub>2</sub>EDTA solution should be stored in a refrigerator or cooler at 4° C. Immediately before sample analysis, 8.75 grams of L-ascorbic acid (preweighed in vials) was added to each 250 ml of the NaOH-EDTA solution. The SAOB buffer solution is stable for up to 3.0 hours after addition of L-ascorbic acid. As 5 ml of this solution was used for each sample analyzed (described below), 250 ml is sufficient for 50 samples.

Stock and diluted S<sup>-</sup> solutions used as electrode calibration standards should be kept cool (~4 °C) and diluted just before use. As with NH<sub>4</sub><sup>+</sup> standards, dilute S<sup>-</sup> standards are unstable when exposed to air. Diluted standards are stable for up to 3 hours, thus they should be made up in the morning and at mid-day on the day of sample analysis. The S<sup>-</sup> electrode was calibrated before and after each batch of not more than 12 samples. Three S<sup>-</sup> standards (100, 1000 and 10,000 µM) were typically used for a three-point electrode calibration. A stock S<sup>-</sup> solution of 0.01 M Na<sub>2</sub>S is prepared by weighing 0.2402 g Na<sub>2</sub>S.9H<sub>2</sub>O into a Biotight jar and diluting to 100 ml with distilled water. Na<sub>2</sub>S.9H<sub>2</sub>O is hygroscopic and should be handled with rubber gloves in a fume hood. The solution should be made fresh every 48 hours and stored at 4° C in a dark bottle. A 1000 µM S<sup>-</sup> standard (10<sup>-3</sup> M) was prepared by transferring 10 ml of the 0.01 M Na<sub>2</sub>S stock solution (10,000 µM) into an amber jar and diluting to 100 ml with distilled water. A 100 µM S<sup>-</sup> standard (10<sup>-4</sup> M) was prepared by transferring 10 ml of the 1000 µM standard to an amber jar and diluting to 100 ml with distilled water. Both dilution standards were mixed thoroughly before use. A two or three-point calibration procedure was performed following the meter's instruction manual. Just before calibration of the S<sup>-</sup> electrode, 25 ml of each standard was transferred to a dark bottle and 25 ml of SAOB (containing ascorbic acid) added. The combined solution was kept tightly capped until used for standardizing the S<sup>-</sup> electrode.

**3.2.10.2. Measurement of sediment total sulfides.** Following the measurement of redox potential, the sample container's lid was removed, and the sample was homogenized using a stainless steel spatula. When working with fine-grained sediments, a 5-ml subsample was extracted using a cut-off syringe. The subsample was expelled into a 30-ml graduated beaker. Five ml of the previously described SAOB buffer (to which L-ascorbic acid has been added) was added to the 5-ml subsample for sulfide determination. This technique does not work in coarse sediments (sand, etc.). Analysis of coarse-grained sediments was accomplished by pipetting 5 ml of SAOB buffer to a 30 ml graduated beaker and then adding sediment to the 10 ml mark.

A flat-tip stainless steel spatula was used to mix and homogenize the sediment sample with the SAOB buffer. Following this, the S<sup>-</sup> electrode was used to further stir the sediment. The S<sup>-</sup> electrode reading usually stabilized in two to four minutes.

Electrodes need not be cleaned or recalibrated between analyses of sediments from the same station. However, after completing 12 analyses, the electrode should be gently rinsed with distilled water and recalibrated before continuing. The sulfide electrode should be recalibrated at least once every two hours and at the end of each batch of samples.

**3.2.10.3. Quality assurance for sediment total sulfide analyses.** Each sample should be thoroughly homogenized in the laboratory before a subsample was taken for this analysis. Laboratory homogenization shall be conducted even if the samples were homogenized in the field. Triplicate analyses shall be conducted on one of every 20 samples, or on one sample per batch when fewer than 20 samples were analyzed. The Data Qualification Control Limit is 20% Relative Percent Difference (RPD). Fresh standards were made up daily. The analytical balance was inspected daily and calibrated at least one per week. For work in the

field at remote locations, the 2 N NaOH basic solution was made up prior to deployment. Other ingredients were premeasured into dark vials and used without further measurement in the field.

**3.2.11. Benthic Infaunal Analysis.** The entire contents of infaunal grab samples (including the overlying water) shall be washed from the grab into a five-gallon bucket using 50 µm filtered seawater. Samples are then sieved on 1.0 mm stainless steel screens. The retained material will be placed in 1.0 to 2.0 liter HDPE bottles and fixed using 10% buffered formalin in seawater. Each jar shall have matching inside, outside and cap labels. Fixed samples are washed with filtered fresh water and preserved in 70% isopropyl alcohol within 5 days of fixing.

The sieved and preserved infaunal samples will be sorted and identified by recognized taxonomists. Infaunal organisms should be sorted from the background matrix under 10x magnification using a dissecting microscope. Twenty percent of each sample must be repicked by a different technician. As an alternative, ten percent of the number of samples picked by each sorter should be repacked, in their entirety by a second technician. Quality assurance guidelines require a picking efficiency of >95%. Any sample failing this QA benchmark will be completely repicked.

Infauna shall be identified to the lowest level practicable – generally to species. Data will be entered into an Excel™ spreadsheet for analysis using Statistica™ software. All species will be compared with verified specimens in Aquatic Environmental Sciences' reference collection – or will be verified by an outside expert.

**3.2.12. pH.** Water and sediment pH are measured in the laboratory using a three point (pH 4.0, 7.0 and 10.0) calibrated Corning Model 32 pH meter equipped with a Corning “3 in 1” Combination pH electrode. The instrument is recalibrated after ten measurements and at the end of each batch.

**3.2.13. Temperature.** Temperature will be measured using the temperature mode of the YSI Model 57 dissolved oxygen meter. The probe is calibrated monthly against a certified mercury thermometer.

**3.3. Task A3. Developing benthic invertebrate baseline data in the area of the proposed North Totten mussel farm.** Aquatic Environmental Sciences has completed a preliminary inventory of benthic invertebrates at the North Totten Inlet site. This inventory will be expanded to include 12 samples collected along two orthogonal transects (6 on each transect) crossing in the center of the proposed farm site. Samples will be collected in November 2001. This will characterize the benthic community prior to the start of production. Each of these sediment samples will also be evaluated for sediment grain size, total sulfides, total volatile solids, pH, and oxidation-reduction potential as described in Section (3.2). This inventory will be valuable in defining the resources that would be put at risk should biodeposits exceed the assimilative capacity of local sediments. The inventory will also provide baseline data against which to assess physicochemical changes associated with the mussel culture operation.

**4.0. Task D. Escapement and propagation of mussels.** Brooks (2000) provided a discussion of the genetics of *Mytilus edulis galloprovincialis* and its interaction with other members of the mytilid sibling complex. The following tasks will be implemented in providing Totten Inlet specific information with respect to this question. This part of the study will be delayed until July 2001 pending review of Brooks (2000) by Thurston Counties independent reviewers.

**4.1. Task D1.** Visually search existing mussel populations in Totten Inlet to include intertidal areas and man-built structures such as buoys, piers and floats for mussels morphologically resembling *Mytilus edulis galloprovincialis*. The genetic identity of 90 (three gels) of these mussels will be determined electrophoretically using procedures described in Brooks (1991). This survey will be conducted by boat within three hours either side of low tides of -1.9' MLLW (1243 on Thursday, July 5, 2001) and -1.8' MLLW (1354 on Friday, July 6, 2001). The mussels will immediately be electrophoresed. Intertidal areas immediately adjacent to the proposed North Totten mussel will receive extra attention to define the preproduction mussel population – including photodocumentation of the intertidal area.

**4.1.1. Mussel electrophoresis procedures.** *Mytilus edulis galloprovincialis* standards will be obtained from Taylor Resources broodstock. *Mytilus edulis trossulus* standards will be obtained from the Hood Canal floating bridge and/or Salisbury Point.

A small piece of digestive gland and another of posterior adductor muscle will be minced with forceps in 0.5 ml of tris-HCL (pH 8.0) grinding buffer and placed in pre-numbered, 1.5 ml microfuge tubes frozen into a block of ice. After mincing, the tissues are disrupted using a Fisher Scientific Sonic Dismembrator 60™. The instrument uses a 1/8" probe and is set at 45 watts for 15 seconds. Tissues are then spun down at 2,000 g for 10 minutes and the supernatant absorbed onto two mm wide wicks for horizontal electrophoresis in a 12.5% starch gel.

These gels are run in tris-maleic acid buffer for six hours at 7.9 V/cm at 4°C. Staining procedures are described in Abersold *et al.* (1987). Stains will be mixed within one hour of use in 2% agar for application to a freshly sliced gel. Stained slices will be incubated at 37°C for a period sufficient to reveal the PGM-2 allele (45 minutes to two hours).

Scoring is accomplished by overlying the gel, inside the gel mold, with a glass plate. The enzyme locations are carefully transcribed to an acetate overlay providing a permanent record. Digital photographs of each gel will also be recorded. Scoring is based on the relative mobility of PGM-2 with respect to *Mytilus edulis galloprovincialis* and *Mytilus edulis trossulus* standards, which are run in outside lanes. Brooks (1991) demonstrated that the PGM-2 locus meets the requirements of Avise (1974) to be considered diagnostic for these two siblings and their hybrids.

**4.2. Task D2. Establish three permanent genetic study sites in Totten Inlet.** Three permanent genetic study sites will be established at those locations where the highest numbers of *Mytilus edulis galloprovincialis* and hybrids with *Mytilus edulis trossulus* were observed in Totten Inlet during the surveys described in element 4.1.1. A photographic record of these sites and interim report of the field survey and electrophoresis will be supplied to Thurston County. The county, and its independent reviewers should approve the location of these permanent genetic study sites prior to additional work. If the sites are on private property, then written permission from the property owner will also be required. It is likely that these permanent study sites will be used in anticipated studies required as a condition of the Section 10 permit issued by the U.S. Army Corps of Engineers.

A three-meter square grid will be laid out over the beach with the highest density of mussels at each study site. The corners of each grid will be marked by pounding 1/2" steel rebar into the beach and securing a coded piece of 1-1/2" diameter PVC pipe to the end protruding from the substrate. The GPS coordinates of each site will be recorded.

**4.3. Task D3. Determine the proportion of mussels carrying *Mytilus edulis galloprovincialis* alleles at each of the three permanent study sites.** This task will be completed using the following procedures:

**4.3.1. Random sample collection.** A three meter square PVC pipe grid will be laid out with one corner at each of the pieces of rebar set in accomplishing Task D2. The PVC grid is marked in 10 cm intervals along each side. A 0.1 m<sup>2</sup> quadrat is used to collect three random samples from within this grid. Sample locations are determined by assigning two random numbers, each between one and thirty, corresponding to *x* and *y* coordinates on the PVC pipes. The origin of this coordinate system is the southwest rebar stake defining the sampling area. The numbers define the position of the sample from the origin in tens of centimeters. The 0.1m<sup>2</sup> quadrat is centered on this position and all mussels removed from within the quadrat. A total of 10 mussels per quadrat are required. If 10 mussels are not obtained, then a 1.0 m<sup>2</sup> quadrat will be used. Three samples are collected using identical procedures (with different sets of random numbers) at each study site. A total of 30 mussels per study site are required. The retrieved mussels are gently washed and placed in one-gallon Ziploc™ bags that are held at 4°C until analyzed within 48 hours.

**4.3.2. Sample processing.** Mussels from each quadrat will be gently washed. The length, width and depth of their valves will be measured to 0.1 mm using electronic calipers. The live weight of each mussel will be determined to 0.1 mg. Electrophoresis will be conducted as described in Section 4.1.1 of these protocols. The valves of all mussels will be numbered, air dried, and archived for two years.

**5.0. Summary of activities.** This study is summarized in Table (1) by date – including crew and boat identifications. Weather and/or workload may require modification of the exact dates. However, tidal exchange requirements severely limit the days on which this work can be accomplished. Datasheets identifying the samples and data needed on each sample day are provided with these protocols.

**Table 1. Preliminary sample dates and activities together with crew and boat assignments for Taylor Resources mussel farm EIS studies at the Deepwater Point mussel farm and at the proposed North Totten farm site. Note, use the North Totten site as a reference site for Deepwater to obtain additional information wrt the new site.**

Date	Site	Tide	Crew	Boat	Sediment		Infauna
					Canisters	Sediment Chemistry	
September 2001	Deepwater		B, S, K	Taylor	4 in	12	
November 2001	Deepwater North Totten		B, S, K, C	AES	4	12 12	12 - 0.1 m <sup>2</sup> van Veen 12 - 0.1 m <sup>2</sup> van Veen
January 2002	Deepwater		S, K	Taylor	4	12	
March 2002	Deepwater		S, K	Taylor	4	12	
May 2002	Totten Inlet		B, K	Taylor	Baseline mussel surveys of Totten Inlet for <i>Mytilus edulis galloprovincialis</i> . Collect 180 mussels = 6 gels)		
May 2000	Deepwater		S, K	Taylor	4	12	
July 2002	Deepwater		S, K	Taylor	4 out	12	

1. Canister studies require 4 each one liter HDPE bottles plus 500 ml of 60 ppt saltwater with 15% buffered formalin each day.
2. Infaunal samples require 45 each one liter wide mouth bottles for sampling in November 2000

Deepwater Point sediment and canister datasheet. Sample date \_\_\_\_\_ Number of rafts \_\_\_\_\_ Number lines per raft \_\_\_\_\_  
 Weather \_\_\_\_\_ Air temp \_\_\_\_\_ Surface temp \_\_\_\_\_ Surface salinity \_\_\_\_\_ Surface water turbidity \_\_\_\_\_  
 High tide \_\_\_\_\_ @ \_\_\_\_\_ Low tide \_\_\_\_\_ @ \_\_\_\_\_ Current direction. \_\_\_\_\_  
 Chosen sampling transect bearing from farm \_\_\_\_\_

Number	Distance	Latitude	Longitude	Depth	D.O.	Temp	Salinity	Infauna	Sulfide	ORP	pH	H <sub>2</sub> S	Bacteria/Infauna
1	Center												
2	P												
3	15												
4	30												
5	45												
6	60												
7	80												
8	100												
9	125												
Rep (2a)	P												
Rep (2b)	P												
<b>Control</b>													
10													
11													
12													

Sulfide probe calibrated (100 & 1000 μmoles) at \_\_\_\_\_. End points at \_\_\_\_\_ were 100 μmole \_\_\_\_\_ 1000 μmoles \_\_\_\_\_

ORP probe calibrated at \_\_\_\_\_.

Notes: (1) Farm drawing on back of page.

**Canisters.**

Canister Number	Bearing	Distance	Latitude	Longitude	Depth	Fixative (ml)	Notes
1							
2							
3							
4							