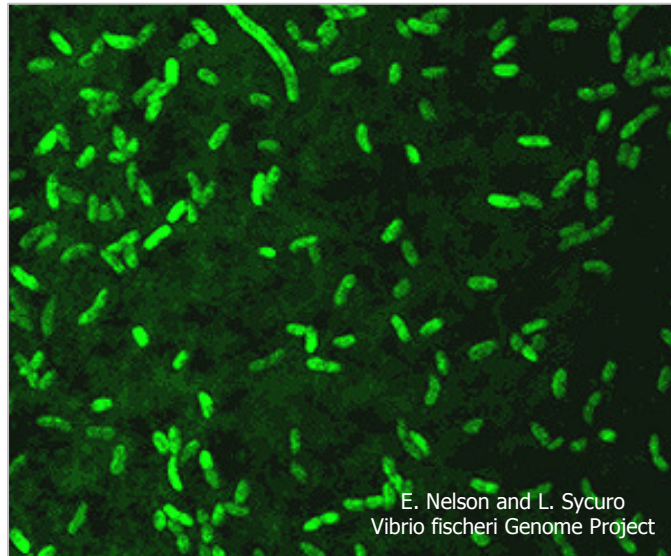


**ABRATOX® PROCEDURE**  
**for Rapid Toxicity Screening and Bioassays**  
**using the bioluminescent bacterium *Vibrio fischeri***

OPTIMIZED FOR USE IN SAN JUAN COUNTY, WA



**Russel Barsh**

KWIAHT (Center for the Historical Ecology of the Salish Sea)  
PO Box 415, Lopez, WA 98261

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**Russel Barsh**

AbraTox® is one of a number of commercially available testing systems utilizing strains of the bioluminescent bacterium *Vibrio fischeri* as bioindicators. In this paper, we describe a modified AbraTox® procedure for evaluating contamination of freshwater and marine sediments in San Juan County, and provide results of a baseline survey of county streams and storm sewer outfalls for sediment toxicity. We also describe a protocol for using AbraTox® to assess the relative toxicity of household products for which relevant environmental-impact data do not exist.

**How AbraTox® works**

The principle of *Vibrio* screening is very simple. Standardized concentrations of the bioindicator organisms are added to buffered environmental samples—usually water, but sometimes sludge or other solids—and incubated for a fixed period of time. At the end of the incubation period, the luminescence of each sample is measured and compared with a negative control. The greater the difference, the greater the “inhibition” caused by the sample: that is, the more toxic the sample is to the test organism.

Testing with *Vibrio* is primarily a “bulk assay” or “screening” method. Instead of focusing on a number of toxic compounds we suspect are in the environment, and testing for them individually—which can be costly and still miss toxics that were not targeted—bulk assays evaluate the aggregate of environmental samples on a single model organism. Bulk assays answer the question, “is the water (or sediment) safe?” rather than answering the question “what is in the water (or sediment)?” Of course, we cannot extrapolate very easily from organisms such as *Vibrio* to fish and amphibians, much less humans. But the strains of *Vibrio* that have been developed for water-quality monitoring respond to toxics at concentrations generally considerably below current EPA drinking water thresholds.

Bioindicator methods are regarded as “screening level” tools because they are not specific—that is, they do not identify *what* is causing the inhibition. Other methods must be used to answer that question.

**Advantages of bulk assays**

The attraction of bulk assays is efficiency: they provide a single, relatively simple and inexpensive measure of the aggregate toxicity of environmental samples that can be used to identify sites deserving closer attention and further investigation. The monitoring method we propose here requires no more than annual sampling, at a cost of less than \$25 per site including replicates for quality assurance.

Measuring bioluminescence requires a specialized, highly sensitive instrument—a luminometer such as the Kikkoman Lumitester®—but otherwise only standard, relatively inexpensive laboratory equipment such as pipettors, a vortexer, and a refrigerator. This is an advantage for local monitoring programs because reliable testing can be done in-house (even by supervised volunteers or students).

### **Concerns and limitations**

Bioindicator methods are not as precise as direct measurements of specific toxics by methods such as liquid chromatography, gas chromatography, and mass spectrometry. Living organisms are never homogeneous; different batches can be more or less sensitive to the same toxic materials. This makes within-series calibration particularly important—that is, comparison of every test result with results of both positive and negative controls tested at the same time in the same run. Inhibition test results are essentially meaningless except relative to simultaneously tested controls. This is why all test results are reported as percent inhibition relative to a negative control such as distilled or nano-pure water that is assumed to have zero inhibition.

Living organisms are also very sensitive to ambient laboratory conditions such as handling, temperature, and light, which can result in false positives, *i.e.* causing inhibition independent of the effects of the environmental samples that are being tested. It would be comforting to be able to assume that daily (even hourly) variations in ambient laboratory conditions are systematic biases that affect every sample in a test run equally, since these variations would also affect the negative controls proportionally, and thus drop out in the calculation of percent inhibition. Unfortunately, this is not the case. Small differences in the amount of time that test tubes or cuvettes are removed from the incubator, exposed to light, or agitated can result in noticeable variations in test results. If two replicates of the same environmental samples are run serially, for example, the 2-5 minute delay between measuring the luminescence of the first set of replicates and the second set of replicates is sufficient to produce lower test results in the second set. Any toxics in the environmental samples—as well as any adverse laboratory conditions—simply continue to affect the test organisms for that additional few minutes.

### **Quality assurance measures**

There are two ways to minimize the risk of false positives when using AbraTox® and similar bioindicator methods. At least two replicates of every environmental sample should be tested simultaneously (as part of the same run, with the same aliquot of *Vibrio*) to account for within-run variations in laboratory conditions. Report the *mean* inhibition of each pair of replicate results, showing the range between them as the error bar around the mean. It is also essential to establish an arbitrary cutoff for treating inhibition results as significant: for instance 60 percent, in which case any mean inhibition results less than 60 percent—or with error bars extending below 60 percent inhibition—are rejected as not significantly toxic. This is a policy matter rather than a statistical question. If the cutoff is simply a trigger for further investigation rather than regulatory intervention, it becomes a question of the level of precaution the agency concerned can afford.

We also recommend at least two replicate samples collected on different dates, to be tested as part of different runs with different aliquots of *Vibrio*, to account for vagaries of sampling and sample-preservation conditions. This is most useful as quality assurance with sediments, since they should not change much chemically between sampling dates a few days to a few weeks separated. Water samples can vary from hour to hour, however, so differences in sequentially collected samples cannot be interpreted as a problem in the test system or the sampling procedure. This is a strong argument for using AbraTox® on sediments: they can be assessed more reliably and accurately than water samples.

Once again, mean inhibition values and error bars can be calculated from replicate samples. A difference between two sequential sediment samples must be interpreted with caution. The longer the delay between the first and second sample collections, the greater is the possibility of an actual chemical change in sediments at the sampling site, such as a toxic “spike” between the first and second sampling date, or dissipation or degradation of toxics that were present when the first sample was taken. It is not unreasonable to regard an apparent decrease in toxicity over time as evidence that a problem, if one existed, is no longer present and does not require follow-up, although it may be more prudent to try to determine whether there is a potential source of non-persistent toxics upstream of the site. Any apparent *increase* in toxicity should be a cause for concern and further investigation. Of course, two sequential results that meet criteria (such as >60 percent inhibition) should be regarded as a confident basis for further investigation at the site.

AbraTox® is sold with pre-mixed positive control and negative control solutions, but we recommend the inclusion of a second positive control, a solution of 10 ppm (10 mg/L) sodium lauryl sulfate (SLS), which reliably results in 96-100 percent inhibition of *Vibrio fischeri*. We find that the pre-mixed, relatively weak positive control often results in hormesis—accelerated growth of the test organism in response to stress—which makes it less than desirable as a standard.

### **Calibrating AbraTox® for San Juan County**

Our preliminary screening of freshwater collected from San Juan County streams, lakes and storm sewer outfalls detected modest levels of inhibition (Appendix IV.A). We then tested sediment samples from many of the same water bodies, and predictably found more widespread and higher levels of inhibition (Appendix IV.B, IV.G). Most persistent bio-accumulative toxics are hydrophobic, and tend to be more concentrated in silts than in the water column—including pyrethroid pesticides, PCBs, and PAHs. Although they may have less impact on fish and amphibians so long as they remain adsorbed to silt, they can re-enter the food chain via benthic detritivores, and their accumulation in sediments should be a cause for concern before they reach detectable levels in water samples.

While AbraTox® is sufficiently sensitive to discriminate between relatively more or less contaminated San Juan County watersheds from current levels of dissolved solids, testing sediments in these watersheds would provide earlier detection of problems.

As noted above, sediments also capture a longer-term signal than water samples—months to years, as opposed to days or hours—and are far less likely to miss brief events, such as chemical spills or sporadic wastewater discharges. We have accordingly adapted the standard AbraTox® method for assaying water, so that it can be used to assay the fine

silt in freshwater and marine sediments without adding significantly to the complexity or cost of testing.

### **Variability in sediments**

Water is routinely treated as a uniform testing matrix that is comparable across all collecting sites. Sediments clearly are not. Separating silts from coarser grains in a sieve is one way of reducing differences between sediments. Collecting similar deposits—such as fine mud from accretion areas rather than sand from areas with higher velocity flows—can also reduce matrix variability. The more uniform the sediments in terms of grain size the more comparable the results of *Vibrio* testing. When dried and crushed, all sediments should look like fine soft brown powder with no visible mineral grains.

Absent major spill events, concentrations of contaminants in sediments should not vary greatly over very short time periods (days to weeks). One means to achieve greater statistical reliability for AbraTox® results is repeated sampling of the same sites over 2-3 weeks, and averaging the results. We recommend this approach.

Over the course of the 30-minute AbraTox® incubation period, coarser sediments drop from suspension, but very fine silts—which have more surface area per unit weight, and carry a greater proportion of adsorbed contaminants—remain in suspension longer. Residual turbidity scatters light, depressing bioluminescence results and confounding the effects of turbidity and contaminants. This effect is mainly a function of grain size rather than grain composition, as we have found by experiment (Appendix IV, Table IV.G), and results in less than 50 percent inhibition except at grain sizes less than 88 microns.

We do not recommend using pure silica, silts or fine sands as controls, since they can vary as much in grain size distribution as different environmental samples, and hence cannot be matched perfectly (even if they can be purchased with guaranteed purity). But if the significance criterion is fixed at 60 percent inhibition (or greater), the confounding effects of residual turbidity are minimized, and purely aqueous controls can be used.

We note that allowing solids to settle for longer after the addition of *Vibrio* is not an alternative because differences in inhibition between specimens narrow and disappear. Centrifugation or filtration removes the test bacteria from the water column together with the silts. While adsorbed hydrophobic contaminants can be removed from the silt with organic solvents such as methanol, and then recovered by evaporating the solvent, they cannot easily be re-dissolved in the medium required by the bacteria (water). If the aim of testing sediments is to capture data on persistent toxics accumulating on silts (rather than dissolved in the water column), the bioindicator organisms must be directly exposed to unmodified silt specimens.

### **Usefulness of marine sediments**

*Vibrio fischeri* is sensitive to salinity, but rinsing fine sediments in a large volume of nano-pure water minimizes this interference. We recommend a ratio of 25:1 water to sediment (Appendix II). Rinsing removes any water-soluble contaminants in pore water, together with sodium and magnesium chlorides and other dissolved inorganic salts, but at neutral pH and ambient laboratory temperatures leaves hydrophobic adsorbed compounds

largely unaffected. The possibility of a small amount of residual salinity should be borne in mind when comparing test results for marine and freshwater sediments, however.

Fine freshwater sediments are relatively easy to locate, in pools and side-channels where currents slow and form accretion pockets, and they can only originate upstream in the same watershed. Marine sediments are more mobile and tend to drift along shorelines and down-slope into deeper basins. Inter-tidal and shallow-water collections may reflect combinations of local inputs, drift from hundreds (if not thousands) of meters away along the shoreline, and upwelling from deep sediment reservoirs. Only deep, highly stratified marine sediments are unquestionably undisturbed and unmixed. However, the dilution of local contaminant signals by mixing is a function of circulation. In small, low energy bay habitats, local-source sediments tend to predominate, and can be used reliably to monitor total freshwater and terrestrial inputs from the associated uplands (see Appendix IV.E).

### **Corroborating results**

One simple method for corroborating sediment-toxicity results is to extract all of the adsorbed organic compounds from aliquots of the same dried, crushed silt specimens; gently evaporate the solvent at ambient temperature to 50°C; and then test the residue for several locally widespread hydrophobic contaminants such as pyrethroid pesticides. We recommend extracting 1.0 gram of dry crushed sediment in 5.0 mL spectro- or analytical-grade hexane. Swirl the sediment and hexane together in a 25-mL beaker, allow to settle for 5 minutes, pour off the supernatant liquid into a glass vial, cap the vial tightly and let settle overnight. Transfer the clear supernatant liquid into another glass vial and allow it to evaporate in a clean well-ventilated environment such as a fume hood. This will take a few hours. For immunoassays (ELISA), re-dissolve the dry residue in 1.0 mL of a 50:50 mixture of spectro-grade methanol and nano-pure water. The resulting solution is enough for four ELISA tests and should be used within 24 hours. When handling hexane or other organic solvents, use *only* glassware.

Dried crushed silt specimens can also be extracted in buffered acetic acid with pH of 4 to 5, for colorimetric tests of relatively hydrophilic contaminants such as phenols and surfactants. Use spectro (analytical) grade acetic acid buffered with sodium acetate; start with a 5 percent solution of glacial acetic acid and add buffer to the desired pH. A gram of dried crushed silt can be extracted in 10 mL acetic extracting solution. Remember to back-calculate the test results by multiplying by 10, since 1 gram is equivalent to 1 mL.

Silt specimens should be kept frozen at -20°C or lower when not in use, and it should be borne in mind that some contaminants will degrade slowly over days to weeks even at that temperature.

### **Sampling management**

The reliability and usefulness of any environmental monitoring method ultimately depends on collecting truly representative environmental samples. Water, and to an even greater degree sediments can be very patchy in time and space. Toxics are not only being deposited continually, and degraded continuously, but their distribution is affected by the drift and agitation of the matrix. Water bodies stratify and re-mix, sediments accumulate in some places and erode away from others. We strongly recommend composite samples

to minimize the likelihood of over- or under-estimating toxicity. Combining five samples from the same site significantly improves the reliability of bioindicator methods.

### **Bioassays using AbraTox®**

Bioindicators such as *Vibrio fischeri* have another important use: as a method for evaluating the potential adverse impacts of novel products or active ingredients for which ecotoxicity data are not yet available from agencies such as the Environmental Protection Agency or the European Environment Commission. We include (Appendix III) a method for comparing the toxicity of several unknowns, such as new purportedly “earth-friendly” cleaning products, with a substance of known ecotoxicity such as SLS.

### **Real-world comparability**

We have recommended using AbraTox® to monitor toxic loads in freshwater and marine sediments, and trigger source investigations. Can results be interpreted in relation to salmon, or to the animals that form the base of San Juan County’s aquatic food chains, such as aquatic insect larvae? *Vibrio fischeri* are sensitive to many of the compounds that kill vertebrates (Appendix V), but not necessarily to the same degree. And while there is a growing body of literature comparing the effects of various pollutants on *Vibrio* and other popular test organisms, such as *Daphnia magna* and Gammarid amphipods, these animals are not necessarily the most important elements of San Juan County ecosystems.

Anionic surfactants are widely distributed in San Juan County waters at levels of 0.5 to 1.5 parts per million. We have recommended using 10 parts per million (ppm) of the anionic surfactant sodium lauryl sulfate (SLS) as a standard of comparison in each *Vibrio* test. By experiment, we have found that 10 ppm SLS is sufficient for 100 percent 24-hour mortality of freshwater gammarid amphipods, which form a major pillar of the aquatic food web in San Juan County. *Vibrio* test results may therefore be meaningfully reported as in the following example: “Mean inhibition by sediments from stream X was 84 percent compared to 98 percent for 10 ppm SLS”—understanding that 10 ppm SLS is an order of magnitude greater than the current background level of anionic surfactants in San Juan County aquatic ecosystems.

### **Discussion of San County data**

In the course of evaluating the potential usefulness of using AbraTox® to monitor the health of San Juan County aquatic ecosystems, we tested specimens of water and fine sediments from a wide variety of sites and contexts on Lopez, Orcas and San Juan Island. The results illustrate the merits and limitations of bulk assays with *Vibrio*, and also reflect on emerging contaminant issues that the county should begin to address.

Freshwater specimens (Appendix IV, Table A; Map 1): At least three of the five highest inhibition results (greater than 30 percent) appear plausible and easily explained. Two are ferry terminals, and sediments were collected from the outfalls draining parking and loading areas heavily used by motor vehicles. One (Lopez village) is the outfall of a small urban commercial center’s storm drain system. The other high-toxicity sites are not as easily explained: Garrison and Trout Lake streams flow through relatively low density

rural areas, but may have been affected by agricultural chemicals, spill events, or possibly legacy contamination from past farming practices. The low toxicity result for the Friday Harbor town storm sewer seems counter-intuitive, but it must be remembered that a water sample is a very narrow slice of time, especially in a storm sewer that lacks sediments or pools that trap and re-release contaminants as flow levels change. Indeed, this low result is a cogent argument for seeking broader integrators of contamination over time.

Freshwater sediments (Appendix IV, Table B; Map 2): Sediments were generally more toxic than water collected from the same sites. This is especially conspicuous in the case of the Friday Harbor storm sewer. Hummel Lake, a relatively undisturbed rain-fed glacial lake, was least toxic of the sediments tested, albeit with very high variance. There is a plausible explanation for the high toxicity of the Westcott (Briggs Lake) sediment, in that the Briggs Lake weir had been closed for some time, the stream had not been running more than a trickle, and contaminants may have been accumulating in the pool sampled, to be dispersed with the beginning of fall rains. The False Bay sediment also tested high, and we pursued this result by conducting sampling throughout that watershed.

False Bay watershed sediments (Appendix IV, Table C; Map 3): Eight sources or tributaries of the False Bay stream were sampled, together with the freshwater marsh and tidally-influenced estuary at the bottom of the watershed. The downstream sites were the most toxic, as predicted from the downstream drift of silt. One potential source appeared, at Erickson Lake, which is surrounded by homes: relatively high density. The cleanest of the sediments came from a flooded vegetated slough in a pasture, which could result from dilution as well as remedial effects of this artificial open wetland. Most sediments in the False Bay watershed were only marginally toxic, if we attribute roughly 50 percent of the observed inhibition to residual turbidity as suggested earlier.

Paired freshwater and marine sediments (Appendix IV, Table D; Map 4): This test was aimed at determining whether marine and freshwater sediments are comparable. The freshwater sediments were collected just above the tidal prism, and the marine sediments were collected in the mixing zone. With one exception, marine results were lower. This may reflect greater dilution, the scouring of contaminated fines from the inter-tidal zone, or the somewhat larger grain size of the marine sediments collected (see Appendix IV.G). There is no correlation between marine and freshwater toxicity in the paired samples as a whole. The West Beach sediments are consistent with each other however, suggesting no significant mixing, dilution, or out-transport of silts in this watershed's tiny estuary.

Marine inter-tidal sediments (Appendix IV, Table E; Map 5): We collected these sediments from inter-tidal sites around the rims of two heavily developed bays, one with relatively poor circulation (Fisherman Bay, Lopez) and the other relatively well flushed (Friday Harbor, San Juan Island). As expected, sediments from the low-circulation basin were more toxic to *Vibrio*. Within bays, the most toxic sediments came from lagoons and mud flats where fines accrete; the less toxic sediments came from higher-energy beaches.

Corroborative testing of sediments (Appendix IV, Table F): We extracted weakly hydrophobic compounds from ten dried, crushed sediment specimens with pH=5 buffered acetic acid and tested the resulting aqueous solutions for (1) total cyclic hydrocarbons, by spectrophotometry (absorbance at 254 nm); and (2) total phenols, by spectrophotometry (aminoantipyrine colorimetric method). Natural biological processes produce thousands

of cyclic hydrocarbon species, but in relatively small quantities, and most degrade rapidly in the environment. Significant concentrations of cyclic hydrocarbons in ecosystems are generally anthropogenic in origin (fuels and oils, synthetic pesticides and herbicides, and pharmaceuticals, as well as combustion byproducts). Phenols are rarely present at more than 1 part per million (ppm) unless there is an anthropogenic source. The ten sediments we tested differed relatively little with respect to total cyclic hydrocarbons, and there was at best a very weak correlation between toxicity as measured with AbraTox® and total phenols. On the other hand, some cyclic hydrocarbons and phenols were present in all of the sediments tested, which may explain at least some of the observed *Vibrio* inhibition.

## **Recommendations**

### **Future use of AbraTox®**

Despite its limitations (lack of quantitative precision or specificity), AbraTox® is an inexpensive tool for county employees to screen watersheds periodically for increased (or decreased) overall contaminant loads, using fine sediments as integrators. It can be an economical triggering mechanism for source investigations, and more expensive testing for individual toxic chemical species, whether in-house or by State-certified laboratories. It can also be useful for long-term monitoring of aquatic ecosystem health, particularly if used in combination with periodic testing of the same sediments for a short list of specific toxics such as pyrethroids, anionic surfactants, and arsenic that are already known to pose local threats in San Juan County.

For greatest efficiency, we recommend that San Juan County:

- Identify a minimum of 30 long-term sediment-sampling sites in streams, lakes and ponds representing all large and/or developed watersheds in the county.
- Conduct an annual summer survey of fine-sediment toxicity at these sites with AbraTox®.
- Collect a composite (three separate grabs) sediment sample at each site and re-sample each site three times during each summer survey (=three tests per site each year, with two within-run replicates of each test).
- Adopt 70 percent mean inhibition as criterion for follow-up investigations.

Based on our experience in preparing this report, we estimate that the professional time required for each annual survey (30 sites collecting plus testing) will be 12 days, and the cost of supplies (chiefly the test organism, in AbraTox® kits) will be \$1,000 per year. A trained high school or college student can, with supervision, substitute for an employee of the county or professional subcontractor, since procedures require faithful repetition of simple tasks rather than exercises of scientific judgment. Using volunteers or interns can reduce the cost of the testing program we recommend to less than \$5,000 per year.

### **Future contaminant research**

There are two principal challenges in designing contaminant-monitoring programs with limited resources: (1) minimizing the frequency of sampling without missing patchy

or infrequent toxic inputs and underestimating threats; and (2) minimizing the number of tests carried out on each environmental specimen without missing unexpected or unusual toxic species. Combining a bulk or screening-level test such as AbraTox®, with tests for a modest number of threats considered locally relevant on the basis of previous research, addresses the second challenge. The second challenge is addressed by choosing a matrix (such as fine sediments) that captures as much information as possible over time. For the toxicity screening protocol described in this report, fine freshwater sediments are optimal in San Juan County. For specific testing (pyrethroid pesticides, for example), sediments can also be used, since hydrophobic toxic species can be extracted easily from sediments with methanol, and methanol is a suitable matrix for ELISA methods (Barsh et al. 2008).

Specific testing with ELISA also offers an alternative means of capturing data for longer time periods, from months to years: extraction of contaminant-rich lipids from the animals that live in sediments, using methanol. Sedentary attached or burrowing animals such as bivalves (mussels, clams) are more stationary than fine sediments, but they ingest sediments when feeding and tend to accumulate lipophilic molecules adsorbed to silt and sand particles. This makes them reliable indicators of local chemical conditions over the course of their lives (e.g. Gagnon et al. 2004; Chung et al. 2007).

Different species accumulate different toxic compounds at quite different rates, so species should be chosen with particular target analytes in mind, and comparisons should be made within species and not between species (Wentz et al. 1998). It should also be borne in mind that bivalves undergo seasonal metabolic cycles that can affect toxic loads and stress response to those loads, so that sampling must be carried out at the same time each year (Bocchetti et al. 2008). Evidence that a particular species actually accumulates target toxic compounds in proportion to their concentrations in sediments should precede its consideration as a bioindicator or sentinel species (e.g. Moganti & Richardson 2008).

The additional processing required of animal tissue as opposed to sediments raises costs but does not pose significant technical issues. Fresh tissue must be kept frozen until it can be homogenized in a Waring blender with methanol, filtered through a non-reactive ceramic vacuum sieve and resin column, and finally concentrated in a rotary or vacuum evaporator at a temperature just high enough to volatilize the methanol solvent but not the target analytes. This procedure adds 24-36 hours to processing of each composite sample but can also raise the sensitivity of ELISA tests by an order of magnitude or greater—that is, from 50-500 parts per trillion to as little as 1 part per trillion. Lowered detection limits are a primary consideration in choosing between sediments and organisms as test media.

Since toxic loads in animals within a single population can vary considerably, not solely as a function of their age, relatively large samples from each site are indicated: 12-25 at a minimum. This restricts sampling to species and sites where the periodic removal of this many animals is sustainable. In San Juan County, a number of bivalves are rarely, if ever harvested, and are widely distributed and locally abundant, e.g. native bent-nosed clams and (usually in the same soft marine and estuarine sediments) invasive varnish, or mahogany clams. Our laboratory has also observed both native and invasive “pea clams” (Pisidiidae) in freshwater sediments of San Juan County, thus far in five watersheds. This presents the possibility of employing a combination of marine and freshwater clams such as *Nuttallia obscurata* and one or more locally abundant *Pisidium* species as indicators of changing local environmental loads of ELISA-testable toxics such as pyrethroids, 2,4-D,

other pesticides or herbicides, anionic surfactants, and pharmaceuticals—as well as toxics imported to San Juan County by winds and currents such as PCBs and PBDEs.

We recommend a San Juan County pilot study of: (1) sustainable source sites for marine and freshwater indicator bivalves; and (2) loads of pyrethroids, 2,4-D, and PCBs in composite samples from 5-10 source sites; to determine whether periodic testing would be feasible and useful as a complement to annual bulk toxicity assays. Total cost of such a pilot study need not, in our opinion, exceed \$7,500.

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## **Appendix I**

### **GENERAL LABORATORY GUIDANCE**

#### **Storing supplies**

Each box of AbraTox® contains six aliquots of *Vibrio fischeri* as well as positive and negative controls, and reagents for reconstituting the test organisms and buffering the test media. The *Vibrio* cultures have been freeze-dried and are very sensitive to moisture. Keep Abratox® supplies in a dry -20°C (standard) freezer when not in use. Do not allow the vials of Vibrio Reagent to thaw before use. Shelf life: roughly 120 days.

#### **Contamination**

When using AbraTox® or any other analytical technique, it is essential to prevent contamination within the lab, and cross-contamination of specimens. Always observe the *one-way rule*: nothing removed from a stock reagent container goes back into the same container. If more than needed is removed, the excess must be disposed of properly.

#### **Clean-up**

The contents of test tubes and cuvettes should be poured into a waste beaker and treated with 2 mL of bleach (sodium hypochlorite) to kill bacteria. After bleaching, the liquid is safe to go into a septic system or primary sewage treatment system *i.e.* down the drain.

Test tubes and ABRAXIS plastic cuvettes can be re-used after soaking in 15 percent hydrochloric acid and rinsing thoroughly several times in nano-pure water. Disposable 3-mL plastic transfer pipettes and pipettor tips are not re-useable, and should be disposed of as ordinary solid waste.

## **Appendix II**

### **TOXICITY SCREENING with ABRATOX® Optimized for San Juan County, WA**

One box of ABRATOX is sufficient for testing 60 sediment samples. Samples are run in batches of 10. Total operator time (spread over 48 hours) for one batch is roughly five hours.

#### **Sampling sediment**

Collect and combine five 50-cc sediment samples at each site, separated from one another by at least five meters. Use an aluminum or Teflon scoop to skim the uppermost 2 cm of sediments into a clean glass jar with a non-reactive lid liner, and mix thoroughly. Keep jars refrigerated at 4°C until use. Maximum holding time: 24 hours.

#### **Materials for sample preparation**

- Mettler or similar electronic balance accurate to  $\pm 0.01$  grams
- Weigh paper and non-reactive metal spatula for use with the balance
- Standard centrifuge capable of at least 6,000 rpm
- Conical screw-cap 50 mL centrifuge tubes
- Ceramic mortar and pestle
- Ceramic filter funnel (0.5 mm) and large ceramic spatula
- Plastic petri dishes (10) with matching aluminum foil dishes (10)
- Incubator capable of sustained 40°-50° C heating
- Two glass 500-mL beakers
- Nano-pure (double distilled or Milli-Q) water

#### **Screening and de-watering sediments**

Transfer 50 cc of each combined sample into a clean glass beaker and add nano-pure water to 100 mL. Shake the contents vigorously for 10 seconds; then pour the slurry through a ceramic filter funnel into a clean glass beaker, stirring if necessary with a clean ceramic or aluminum spoon. Quickly pour the filtered slurry into a 50-mL plastic conical avoiding any coarse material, cap tightly, and centrifuge for 3 minutes.

Decant the liquid completely, wiping the mouth of the conical with a paper towel to catch the last drops, then use a clean non-reactive spatula to transfer as much of the solids as possible into a 4-cm aluminum foil dish (made from foil as needed) marked with the sample number, and placed in an incubator set at 40-50°C to dry overnight. When the

foil dishes are removed from the incubator place them in clear covered plastic petri dishes for storage at  $-20^{\circ}\text{C}$  until for up to several days before use.

For marine sediments, do not remove the solids after centrifugation, but instead re-suspend them in nano-pure water, and centrifuge again. This extra step should reduce the salinity of the sample to the point that it will not significantly affect the test organism. Marine sediments should nevertheless only be compared with other marine sediments, as salt removal may also remove a small proportion of the less hydrophobic contaminants.

Remove dry solids from each petri dish and crush in a clean ceramic mortar (wipe with a dry paper towel between samples). Weigh out 0.05 grams (50 mg) of the crushed solids, transfer to a clean ABRAXIS plastic cuvette, and place the cuvette directly in the specially marked Nalgene Labtop cooler. Prepare *two* identical cuvettes for each sample.

### **Lab set-up for testing**

Move the AbraTox® Positive Control, Negative Control, Reconstitution Solution, and Osmotic Adjusting Buffer from the freezer to the refrigerator, leaving enough time for them to thaw completely (3-4 hours).

Place the Nalgene Labtop Cooler in the refrigerator at the same time, if it is not already stored there. Once thoroughly chilled, it will maintain a temperature of  $15^{\circ}\text{C}$  for several hours outside the refrigerator.

Tape the specially marked color template for Toxicity Screening (attached) on the lid of the Labtop Cooler, and load the cooler as marked with 20 sediment-filled ABRAXIS plastic cuvettes. Load the controls (columns 7 and 8) with empty cuvettes, and place one empty glass test tube in position A6 for preparation of the Positive Controls.

This procedure uses the Positive and Negative Controls supplied by ABRAXIS, a Blank (all of the reagents but no *Vibrio* bacteria), and a Standard consisting of 10-ppm sodium lauryl sulfate (SLS). The standard provides a real-world comparison reflecting current water quality conditions in San Juan County.

Also assemble on the bench-top with the specially marked Labtop Cooler:

- Two clean 3-mL disposable graduated plastic transfer pipettes
- SL-1000 pipettor set to 800  $\mu\text{L}$  (080) with 4 disposable 1000- $\mu\text{L}$  low-force pipette tips
- PR-200 pipettor set to 100  $\mu\text{L}$  (100) with 3 disposable 200- $\mu\text{L}$  disposable pipette tips
- At least 150 mL of nano-pure water (Milli-Q)
- Stock solution of 10 ppb sodium lauryl sulfate (SLS)
- Plastic beaker for discarded pipettes and pipette tips
- Plastic beaker for discarded test solutions
- Lab bench timer set to 30 minutes
- Variable speed vortexer

- Kikkoman Lumitester® luminometer and plastic cuvette holder
- Data sheet for recording luminescence measurements (attached)

### **Step 1: Preparation of controls (3 minutes)**

Keep cuvettes in the specially marked cooler except when handling them.

Using the SL-1000 pipettor and a single pipette tip, gently add 800 µL of nano-pure water to each of the plastic cuvettes in the cooler *except* the two Positive Controls, the two Negative Controls, and the two Standards.

Using the same pipettor and pipette tip, transfer 800 µL of the Negative Control solution supplied by Abraxis to each of the Negative Control cuvettes, and then to add 800µL of the Standard solution (10 ppm SLS) to each of the Standard cuvettes.

Using the PR-200 pipettor and a fresh pipette tip, transfer 200 µL of the Positive Control solution supplied by Abraxis to the glass test tube. Add 2 mL of nano-pure water to the glass test tube with a plastic transfer pipette and vortex to mix thoroughly. Using the SL-1000 pipettor and a clean pipette tip, transfer 800 µL of this solution into each of the Positive Control cuvettes.

### **Step 2: Re-hydration of the test organism (2 minutes + 30 minutes)**

Using a disposable transfer pipette, add 2.75 mL of Reconstitution Solution to a container of Vibrio Reagent, and swirl for 30 seconds. Incubate the reconstituted Vibrio Reagent in the refrigerator at 4°C for 30 minutes.

### **Step 3: Addition of buffer solution (8 minutes)**

Using the PR-200 pipettor, add 100 µL of Osmotic Adjusting Buffer to each of the plastic cuvettes. Only one pipette tip is necessary, as long as you are careful not to insert it into the solutions in the cuvettes. Vortex each cuvette for 2-3 seconds to mix its contents thoroughly, and return it to its assigned space in the cooler.

### **Step 4: Addition of the test organism (5 minutes + 30 minutes)**

Use the PR-200 pipettor and a single pipette tip to add 100 µL of Vibrio Reagent to each cuvette in the cooler *except* the Blank. Vortex each of the cuvettes to mix the contents thoroughly, and return them to the cooler. Cover and incubate for 30 minutes.

### **Step 5: Measuring luminescence (5 minutes)**

Turn on the Lumitester® and make certain that it is set to Mode 0.

As soon as the timer rings, begin measuring luminescence of each cuvette in order from left to right in the rack. Remove the cuvette with the plastic cuvette holder, place in the well of the Lumitester®, close the lid firmly, and press Enter. The Lumitester® will count down 10 seconds, and then display the measurement. Write each measurement on

the data sheet, remove the plastic cuvette holder, and dispose of the cuvette in the waste beaker. Try to avoid touching the cuvettes, handle them from the top if necessary, and wipe the round bottom of cuvettes with Kim-Wipes to remove fingerprints or smudges.

### **Step 6: Data analysis**

Convert all luminescence data into *percent inhibition* relative to the luminescence of the Negative Controls. If the Negative Control measured 700 on the Lumitester, and a particular sediment sample measured 300, the inhibition is  $(700-300)/700$  or 57 percent.

The two replicates of a single sample may yield somewhat different results. Take the mean of the two results, and show the range between the two test results as an error bar. If the results for a particular sample are 54% and 42%, for example, report the mean as 48% and the error bar as  $48\% \pm 6\%$  (that is, 42%-54%).

Apply the previously established cutoff criterion to the results to determine which samples have exhibited sufficient toxicity to merit re-sampling and further investigation – or to indicate that efforts to remedy previously detected conditions have not succeeded.

## Appendix III

### BIOASSAYS OF PRODUCTS with ABRATOX® Optimized for use in San Juan County, WA

One box of ABRATOX is sufficient for assaying 12 products, two at a time as one batch. Total operator time for one batch is roughly four hours.

#### Lab set-up for bioassays

Move the ABRAXIS Positive Control, Negative Control, Osmotic Adjusting Buffer, and Reconstitution Solution from the freezer to the refrigerator, leaving enough time for them to thaw completely (3-4 hours).

Place the Nalgene Labtop Cooler in the refrigerator at the same time, if it is not already stored there. Once thoroughly chilled, it will maintain a temperature of 15°C for several hours outside the refrigerator.

Tape the specially marked template for Bioassays (attached) on the lid of the Labtop Cooler, and load the cooler as marked with 28 clean ABRAXIS plastic cuvettes. Place one empty glass test tube in position A6 for preparation of the Positive Controls.

This procedure uses the Positive and Negative Controls supplied by ABRAXIS, a Blank (all of the reagents but no *Vibrio* bacteria), and a Standard consisting of 10-ppm sodium lauryl sulfate (SLS). The standard provides a real-world comparison reflecting current water quality conditions in San Juan County.

Also assemble on the bench-top with the specially marked Labtop Cooler:

- The two products to be assayed
- Specially marked nylon test tube rack with 10 clean glass 10x75 test tubes
- 100-mL glass graduated cylinder
- 10-mL glass graduated cylinder
- Five clean 3-mL disposable graduated plastic transfer pipettes
- SL-1000 pipettor set to 800 µL (080)
- 4 disposable 1000-µL low-force disposable pipette tips
- PR-200 pipettor set to 100 µL (100)
- 4 disposable 200-µL disposable pipette tips
- 250 mL of nano-pure water (Milli-Q)
- Plastic beaker for discarded pipettes and pipette tips
- Plastic beaker for discarded test solutions
- Lab bench timer set to 30 minutes

- Variable speed vortexer
- Kikkoman Lumitester® luminometer and plastic cuvette holder
- Data sheet for recording luminescence measurements (attached)

### **Step 1: Serial dilution of analytes (15 minutes)**

This step uses a specially marked “Bioassays” nylon test tube rack, with 10 glass test tubes arranged in two groupings of five tubes. The first tube in each grouping is for the highest concentration of the product to be tested. This can be full strength product as sold, or it can be a dilution (recommended if the product is viscous or cloudy). Carefully note the concentration of the main active ingredient as declared on the product label, as it will be needed to calculate the IC50 of the active ingredient as opposed to the IC50 of the product as a whole.

Using the PR-200 pipettor and a fresh pipette tip, add 100  $\mu$ L of the first product to the large graduated cylinder. Fill the graduated cylinder slowly to 100 mL with nano-pure water for a beginning dilution of 0.1 percent (1 part per thousand or 1000 ppm).

Add 1 mL of this solution to the 10-mL graduated cylinder with a fresh disposable transfer pipette. Fill to 10 mL with nano-pure water for a 100-ppm dilution, and use the same disposable pipette to transfer 2 mL of this solution to the first test tube in the nylon rack (marked *100 ppm*).

Remove all but 5 mL of the contents of the 10-ml graduated cylinder then re-fill it to 10 mL with nano-pure water for a 50-ppm dilution. Use the same disposable pipette to transfer 2 mL of this solution to the second test tube in the nylon rack (marked *50 ppm*).

Remove all but 2 mL of the contents of the 10-ml graduated cylinder then re-fill it to 10 mL with nano-pure water for a 10-ppm dilution. Use the same disposable pipette to transfer 2 mL of this solution to the third test tube in the nylon rack (marked *10 ppm*).

Remove all but 1 mL of the contents of the 10-ml graduated cylinder then re-fill it to 10 mL with nano-pure water for a 1-ppm dilution. Use the same disposable pipette to transfer 2 mL of this solution to the second test tube in the nylon rack (marked *1 ppm*).

Remove all but 1 mL of the contents of the 10-ml graduated cylinder then re-fill it to 10 mL with nano-pure water for a 0.1-ppm dilution. Use the same disposable pipette to transfer 2 mL of this solution to the second test tube in the nylon rack (marked *0.1 ppm*).

Thoroughly rinse the graduated cylinders with nano-pure water, and with a fresh disposable transfer pipette, repeat the same steps for five dilutions of the other product to be tested.

### **Step 2: Transfer to cuvettes (5 minutes)**

Using the SL-1000 pipettor, transfer 800  $\mu$ L aliquots of the serial dilutions in the nylon test tube rack to the corresponding plastic cuvettes in the Labtop Cooler. There are two replicates for each dilution of each product. Use a fresh pipette tip for each analyte. Keep plastic cuvettes in the Labtop Cooler except when handling them.

**Step 3: Preparation of controls (3 minutes)**

Using the SL-1000 pipettor and a fresh pipette tip gently add 800  $\mu\text{L}$  of nano-pure water to each of the two Blank cuvettes.

Using the same pipettor and pipette tip, transfer 800  $\mu\text{L}$  of the Negative Control solution supplied by Abraxis to each of the Negative Control cuvettes, and then to add 800 $\mu\text{L}$  of the Standard solution (10 ppm SLS) to each of the Standard cuvettes.

Using the PR-200 pipettor and a fresh pipette tip, transfer 200  $\mu\text{L}$  of the Positive Control solution supplied by Abraxis to the glass test tube. Add 2 mL of nano-pure water with a plastic transfer pipette and vortex to mix thoroughly. Using the SL-1000 pipettor and a clean pipette tip, transfer 800  $\mu\text{L}$  of this solution into each of the Positive Control cuvettes.

**Step 4: Addition of buffer solution (8 minutes)**

Using the PR-200 pipettor, add 100  $\mu\text{L}$  of Osmotic Adjusting Buffer to each of the plastic cuvettes. Only one pipette tip is necessary, as long as you are careful not to insert it into the solutions in the cuvettes. Vortex each cuvette for 2-3 seconds to mix its contents thoroughly, then return it to its assigned space in the cooler.

**Step 5: Re-hydration of the test organism (2 minutes + 30 minutes)**

Using a disposable transfer pipette, add 2.5 mL of Reconstitution Solution to a container of Vibrio Reagent, and swirl for 30 seconds. Incubate the reconstituted Vibrio Reagent in the refrigerator at 4°C for 30 minutes.

**Step 6: Addition of the test organism (5 minutes + 30 minutes)**

Use the PR-200 pipettor and a single pipette tip to add 100  $\mu\text{L}$  of Vibrio Reagent to each cuvette in the rack *except* the two Blanks. Vortex each cuvette in turn to mix its contents thoroughly and return it to the Labtop Cooler. Cover the cooler and incubate for 30 minutes.

**Step 7: Measuring luminescence (5 minutes)**

Turn on the Lumitester® and make certain that it is set to Mode 0.

As soon as the timer rings, begin measuring luminescence of each cuvette in order from left to right in the rack. Remove the cuvette with the plastic cuvette holder, place in the well of the Lumitester®, close the lid firmly, and press Enter. The Lumitester® will count down 10 seconds, and then display the measurement. Write each measurement on the data sheet, remove the plastic cuvette holder, and dispose of the cuvette in the waste beaker. Try to avoid touching the cuvettes, handle them from the top if necessary, and wipe the round bottom of cuvettes with Kim-Wipes to remove fingerprints or smudges.

### **Step 6: Data analysis**

Convert all luminescence data into *percent inhibition* relative to the luminescence of the Negative Controls. If the Negative Control measured 700 on the Lumitester, and a particular serial dilution measured 300, the inhibition is  $(700-300)/700$  or 57 percent.

The two replicates of a single sample may yield somewhat different results. Take the mean of the two results, and show the range between the two test results as an error bar. If the results for a particular sample are 54% and 42%, for example, report the mean as 48% and the error bar as  $48\% \pm 6\%$  (that is, 42%-54%).

Graph the results for each product tested, and include the best-fit regression curve. Infer the IC<sub>50</sub> (50-percent inhibition concentration) from the regression curve, i.e., where the curve crosses 50% inhibition. Draw a line through the test result for the 10-ppm SLS Standard for visual comparison.

## ABRATOX DATA SHEET: TOXICITY SCREENING

Date: \_\_\_\_\_

Tested by: \_\_\_\_\_

Duration of exposure: 30 minutes

Holding time: \_\_\_\_\_ hours

<i>Specimen source</i>	<i>Bottle</i>	<i>Luminescence</i>		<i>% Inhibition</i>	
		A	B	A	B
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Controls	Positive				
	Negative				
	Blank				
	Standard				

Percent inhibition is the ratio of (Negative Control minus Measurement)/Negative Control)

Unless otherwise specified, the Standard is 10 ppm Sodium Lauryl Sulfate

## ABRATOX DATA SHEET: BIOASSAYS

Date: \_\_\_\_\_

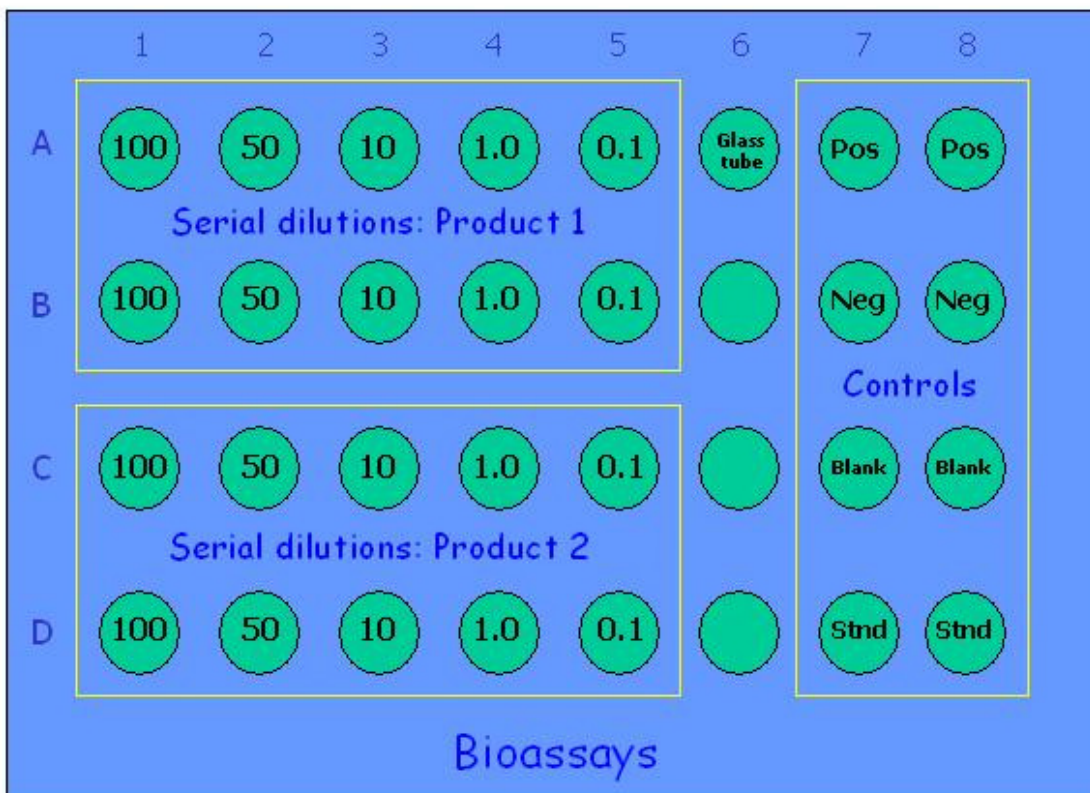
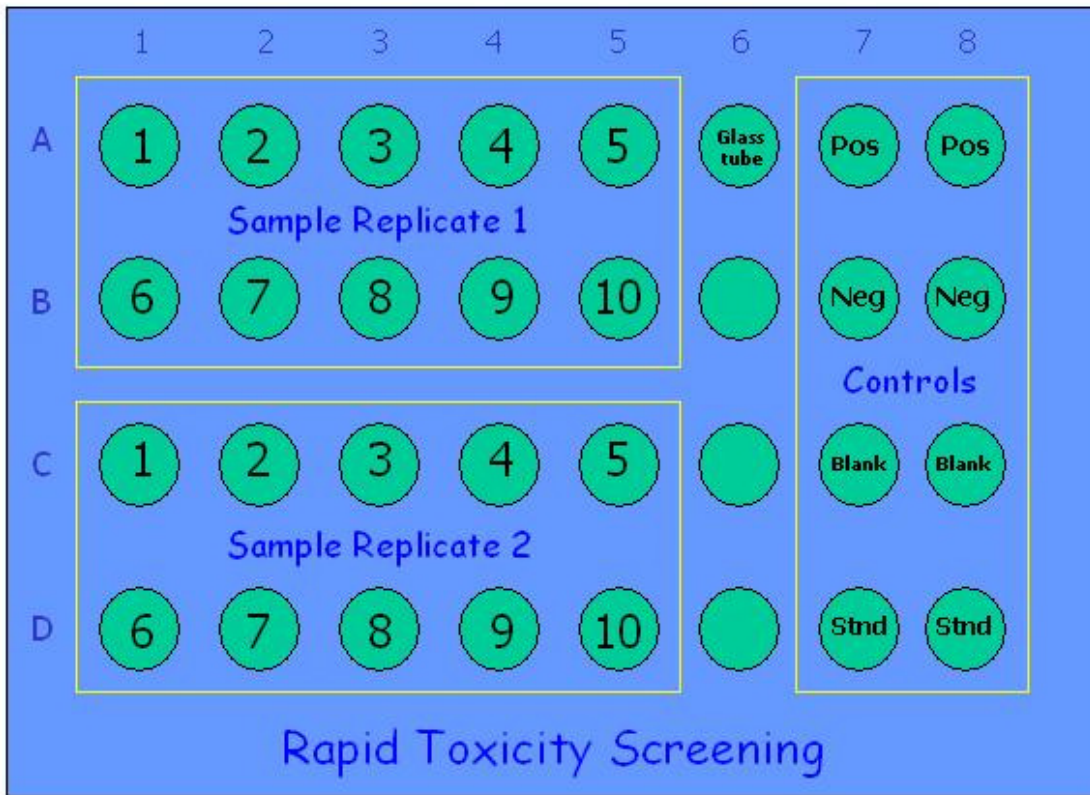
Tested by: \_\_\_\_\_

Incubation time: 30 minutes

Analytes	Dilution ppm	Luminescence		% Inhibition	
		Replicate A	Replicate B	Replicate A	Replicate B
1 <sup>st</sup> Product name:  Active ingredient:  And its concentration:	100				
	50				
	10				
	1.0				
	0.1				
2 <sup>nd</sup> Product name:  Active ingredient:  And its concentration:	100				
	50				
	10				
	1.0				
	0.1				
CONTROLS	Positive				
	Negative				
	Blank				
	Standard				

Percent inhibition is the ratio of (Negative Control minus Measurement)/Negative Control)

Unless otherwise specified, the Standard is 10 ppm Sodium Lauryl Sulfate



## Appendix IV

### REPRESENTATIVE SAN JUAN COUNTY TEST DATA

#### IV.A. Comparison of AbraTox® results for freshwater samples from eight watersheds<sup>1</sup>

Island	Watershed	Sampling site	Context	% Inhibition <sup>2</sup>		
				A	B	Mean
Lopez	Fisherman Bay	Lopez Village swale	Town storm drain outfall to marsh	28	35	<b>32</b>
	Shoal Bay	Lopez ferry dock	Road-parking area storm drain outfall	37	39	<b>38</b>
San Juan	Beaverton Valley	Friday Harbor ferry dock	Road-parking area storm drain outfall	31	34	<b>32</b>
		Friday Harbor storm sewer	Town storm drain outlet to bay	9	7	<b>8</b>
		Beaver Creek (University)	Stream channel 75 m above tidal prism	0	0	<b>0</b>
	False Bay	False Bay marsh	Marsh channel 50 m above tidal prism	20	27	<b>24</b>
	Garrison Bay	Blazing Tree Road	Stream channel above road culvert	26	43	<b>34</b>
	Sportsman Lake	Public boat ramp	Shallow vegetated lake edge	1	9	<b>5</b>
	Trout Lake	Prohaska Road	Stream channel below road culvert	25	34	<b>30</b>
	Westcott Bay	West Valley Road	Stream channel below road culvert	4	25	<b>14</b>

<sup>1</sup> Collected November 3, 2008; tested November 4, 2008. For locations, see Map 1.

<sup>2</sup> Two replicates run simultaneously as a single batch.

#### IV.B. Comparison of AbraTox® results for freshwater sediments from eight watersheds<sup>3</sup>

Island	Watershed	Sampling site	Context	% Inhibition <sup>4</sup>		
				A	B	Mean
Lopez	Fisherman Bay	Lopez Village swale	Town storm drain outfall to marsh	79	88	<b>84</b>
	Shoal Bay	Lopez ferry dock	Road-parking area storm drain outfall	55	84	<b>70</b>
	Swifts Bay	Hummel Lake outlet	Lake outlet (road culvert) to marsh	5	57	<b>36</b>
San Juan	Beaverton Valley	Friday Harbor ferry dock	Road-parking area storm drain outfall	31	70	<b>50</b>
		Friday Harbor storm sewer	Town storm drain outlet to bay	50	75	<b>62</b>
		Beaver Creek (University)	Stream channel 75 m above tidal prism	45	57	<b>51</b>
	False Bay	False Bay marsh	Marsh channel 50 m above tidal prism	78	80	<b>79</b>
	Garrison Bay	Blazing Tree Road	Stream channel above road culvert	56	58	<b>57</b>
	Trout Lake	Prohaska Road	Stream channel below road culvert	68	72	<b>70</b>
	Westcott Bay	West Valley Road	Stream channel below road culvert	96	97	<b>96</b>

<sup>3</sup> Collected November 24-25, 2008; tested November 26, 2008. For locations, see Map 2.

<sup>4</sup> Two replicates run simultaneously as a single batch.

**IV.C. Comparison of AbraTox® results for sediments collected throughout one watershed<sup>5</sup>**

Island	Watershed	Sampling site	Context	% Inhibition <sup>6</sup>		
				A	B	Mean
San Juan	False Bay	Beaverton Valley Road	Roadside seasonal marsh	69	69	<b>69</b>
		Erickson Lake	Marshy lake surrounded by homes	88	96	<b>92</b>
		Egg Lake Road	Farm pond outflow under county road	56	72	<b>64</b>
		Boyce Road	Stream channel above road culvert	54	71	<b>63</b>
		Prohaska Road	Stream channel below road culvert	65	77	<b>71</b>
		Land Bank weir	Stream channel below road culvert	63	66	<b>65</b>
		Zylstra Lake inflow	Marsh below West Valley Road culvert	66	73	<b>70</b>
		Valley Farms Road	Flooding channel in cattle pasture	47	54	<b>50</b>
		University preserve marsh	Marsh channel 50 m above tidal prism	95	97	<b>96</b>
		False Bay estuary <sup>7</sup>	Estuary channel, at low tide	96	98	<b>97</b>

<sup>5</sup> Collected January 15, 2008; tested January 16, 2008. For locations, see Map 3.

<sup>6</sup> Two replicates run simultaneously as a single batch.

<sup>7</sup> Marine-influence sediment, double washed to reduce salinity.

**IV.D Comparison of AbraTox® results for paired freshwater and marine sediments from five watersheds<sup>8</sup>**

Island	Watershed	Sampling site	Context	% Inhibition <sup>9</sup>		
				A	B	Mean
Orcas	Cascade Creek	Taylor's streamside	Wide shady channel, year-round flow	35	63	<b>49</b>
		Buck Bay mudflat	Broad shallow muddy tide flat	46	32	<b>39</b>
	Crow Valley	Margo's Lane	Deep shady instream year-round pool	47	66	<b>56</b>
		Crow Valley estuary	Small & muddy, behind road causeway	42	26	<b>34</b>
	Doe Bay	Pt Lawrence Road	Deep plunge pool below county culvert	75	78	<b>76</b>
		Resort beach	Sheltered shallow beach with fine sand	0	0	<b>0</b>
	Eastsound	Outlook Inn pond	Small dug pond with much algae	97	98	<b>97</b>
		Storm sewer outfall	Soft fine sand-shell beach below culvert	16	0	<b>8</b>
	West Beach	Pottery pond	Large shady still muddy dug pond	98	99	<b>98</b>
		Estuary pool	Small tidally influenced stream pool	88	94	<b>91</b>

<sup>8</sup> Collected January 5, 2009; tested January 7, 2009. For locations, see Map 4.

<sup>9</sup> Two replicates run simultaneously as a single batch.

**IV.E. Comparison of AbraTox® results from inter-tidal sediments of two San Juan County bays<sup>10</sup>**

Island	Watershed	Sampling site	Context	% Inhibition <sup>11</sup>		
				A	B	Mean
Lopez	Fisherman Bay	Weeks wetland slough	Tidal mud flat and slough; urban	86	93	<b>90</b>
		IMC boatyard dock	Modified beach beneath road armoring	82	93	<b>88</b>
		The Galley dock	Modified beach beneath road armoring	30	66	<b>48</b>
		Otis Perkins park	Shallow vegetated tidal marsh	84	94	<b>89</b>
		Fish Bay Spit lagoon	Shallow tidal lagoon with sandy beach	87	96	<b>92</b>
San Juan	Friday Harbor	Friday Harbor Labs dock	Sandy beach below high seawall	46	58	<b>52</b>
		Beaver Creek Cove	Unmodified sandy-rocky beach	75	76	<b>76</b>
		Port of F.H. fuel dock	High bank stabilized with rocks, muddy	89	94	<b>92</b>
		F.H. storm sewer outfall	Sandy-rocky beach below high sea wall	56	59	<b>58</b>
		Jensen's shipyard	Sandy beach, old docks and debris	59	73	<b>66</b>

<sup>10</sup> Collected February 12, 2009; tested February 13, 2009. For locations, see Map 5.

<sup>11</sup> Two replicates run simultaneously as a single batch.

**IV.F Comparison of AbraTox® results with other screening level data (freshwater matrices only)**

Island	Watershed	Sampling site	% Inhibition <sup>12</sup>	% Absorbance <sup>13</sup>	Phenol ppm <sup>14</sup>
Lopez	Fisherman Bay	Lopez Village swale	88	1.8677	6.56
Orcas	Eastsound	Outlook Inn pond	98	1.8303	9.00
	West Beach	Pottery pond	99	2.5914	3.49
	Cascade Creek	Taylor's reach	63	0.9451	4.09
San Juan	False Bay	Erickson Lake	96	2.7092	10.28
		University preserve	97	2.4982	6.64
		Valley Farms Road	54	1.9843	4.56
	Westcott Bay	West Valley Road culvert	97	1.8835	10.36
	Beaverton Valley	University Road reach	57	2.2413	8.68

<sup>12</sup> AbraTox® results from tables IV.B, IV.C, and IV.D.

<sup>13</sup> Preserved (frozen) dry crushed specimens extracted in pH=4 buffered acetic acid and filtrate scanned at 254 nm.

<sup>14</sup> By aminoantipyrine method on extracts prepared as in note 2, after subtraction of reagent blank.

**IV.G. Comparison of AbraTox® results for paired freshwater sediment and water samples<sup>15</sup>**

Island	Watershed	Sampling site	Context	Matrix	% Inhibition <sup>16</sup>		
					A	B	Mean
San Juan	Garrison Bay	Blazing Tree Road	Stream channel above road culvert	Water	0	0	<b>0</b>
				Sediment	52	74	<b>63</b>
		States Inn driveway	Plunge pool below road culvert	Water	71	86	<b>79</b>
				Sediment	49	77	<b>63</b>
	Lakedale Lakes	Resort driveway	Shallow alder shaded dug pond	Water	10	8	<b>9</b>
				Sediment	66	84	<b>75</b>
	Sportsman Lake	Public boat ramp	Shallow vegetated lake edge	Water	1	0	<b>1</b>
				Sediment	14	55	<b>35</b>
	Westcott Bay	West Valley Road	Stream channel below road culvert	Water	0	0	<b>0</b>
				Sediment	80	87	<b>84</b>

The Blacing Tree and West Valley Road water samples exhibited hormesis, which we hypothesized were due to high nutrient loads; colorimetric total nitrate measurements were consistent with this hypothesis.

<sup>15</sup> Collected February 16, 2009; tested February 16, 2009. For locations, see Maps 1 and 2.

<sup>16</sup> Two replicates run simultaneously as a single batch.

Figure IV.A. Comparison of AbraTox® results for freshwater specimens from 8 watersheds

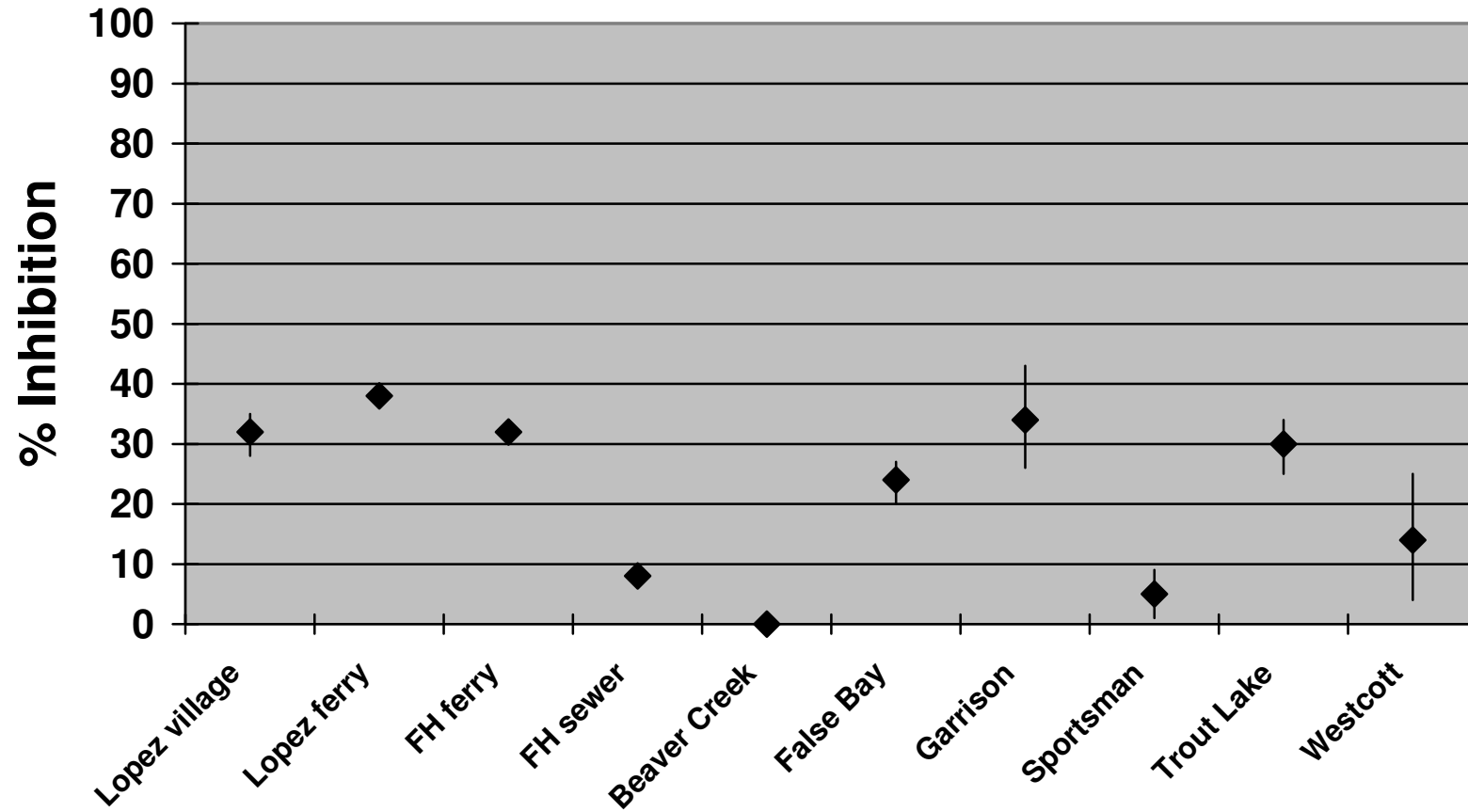


Figure IV.B. Comparison of AbraTox® results for freshwater sediments from 8 watersheds

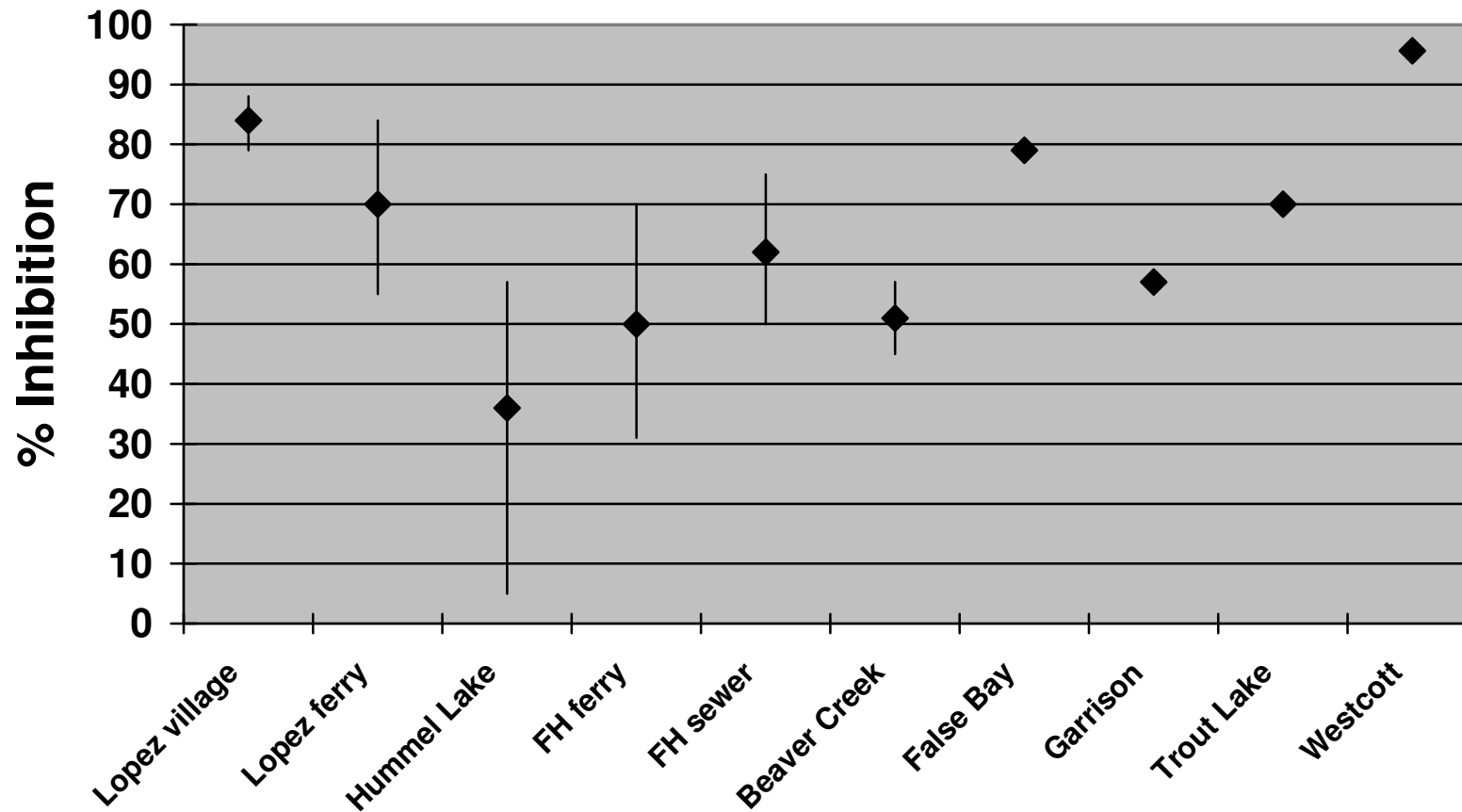


Figure IV.C. Comparison of AbraTox® results for freshwater sediments from False Bay watershed

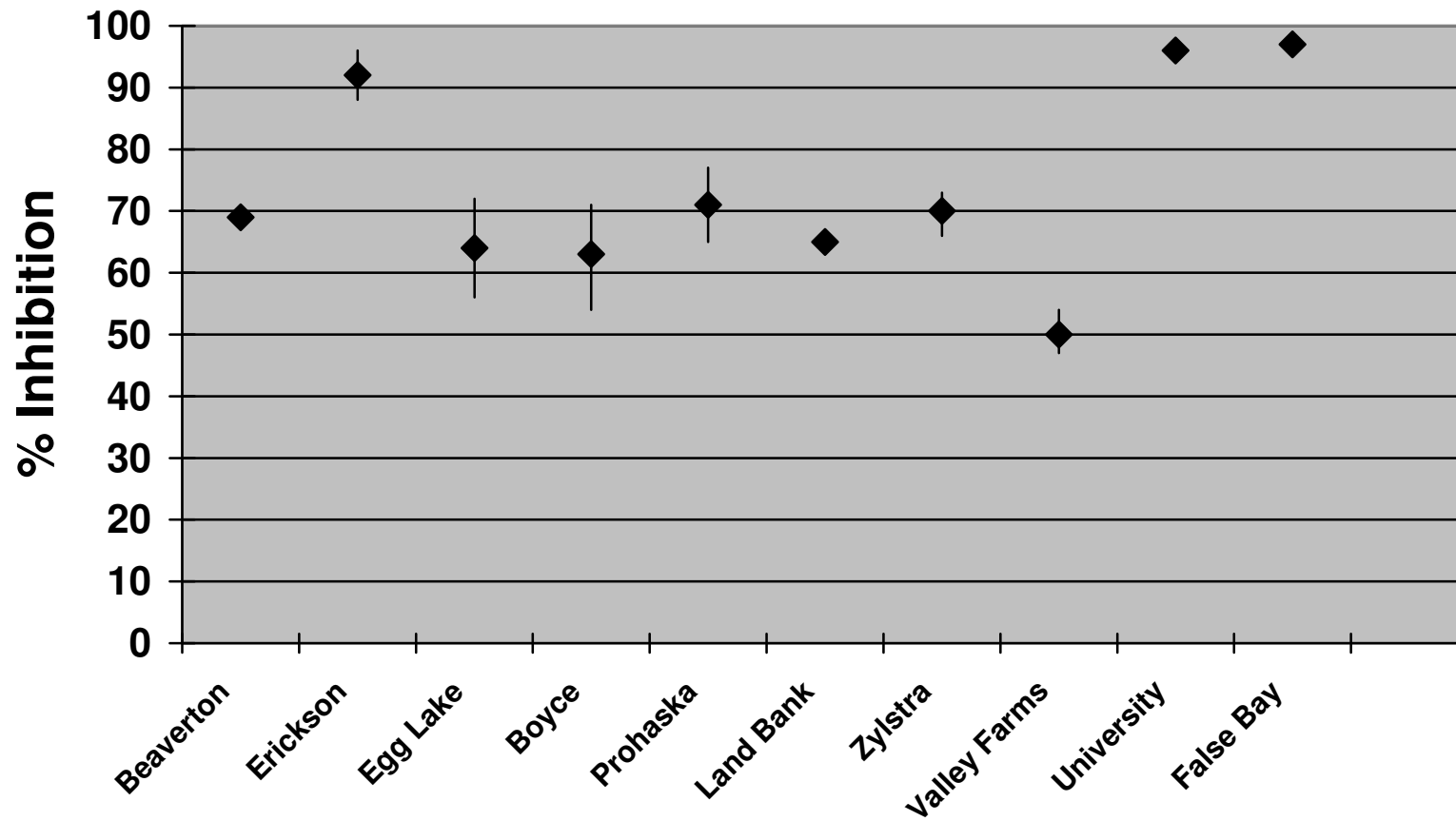
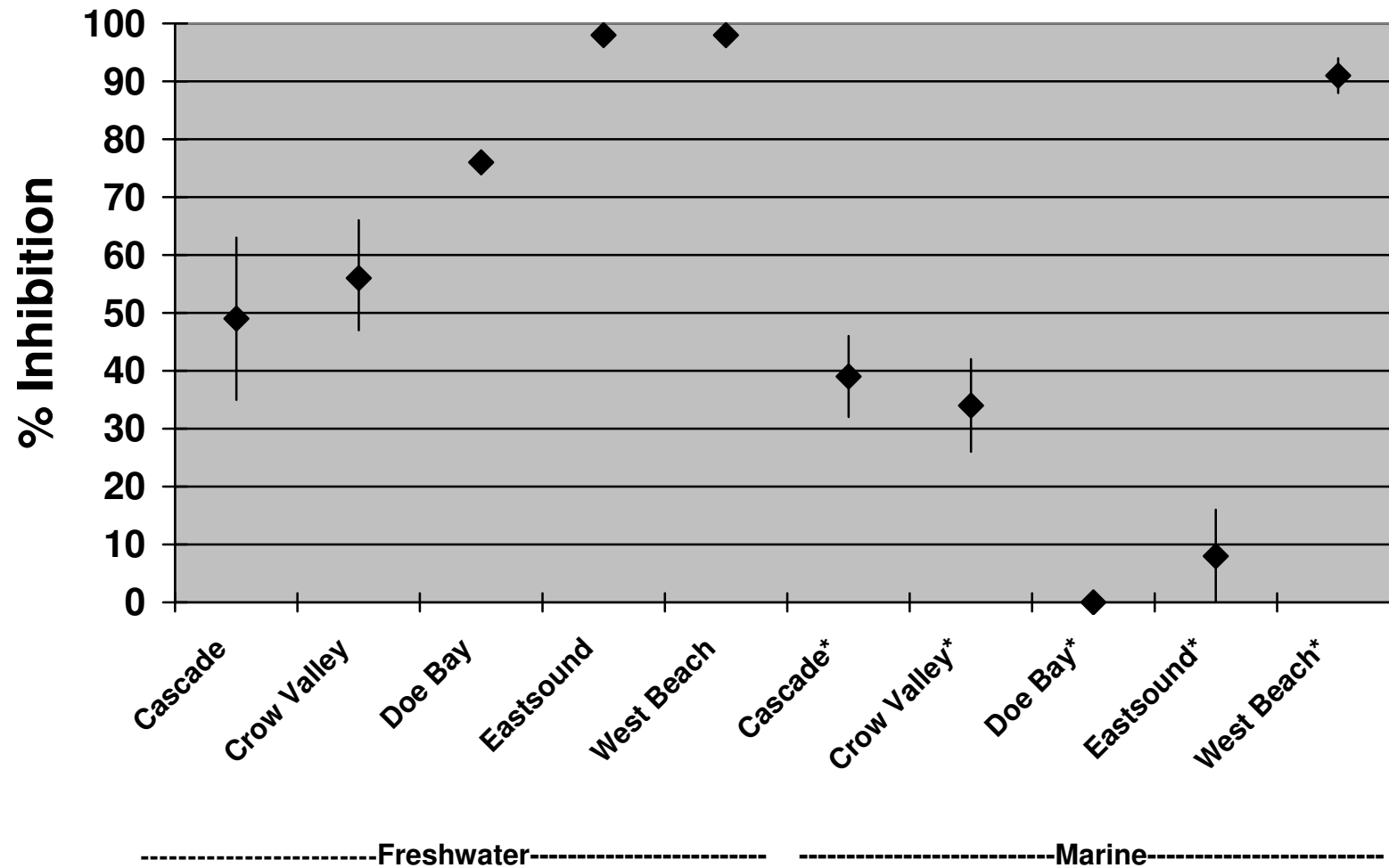
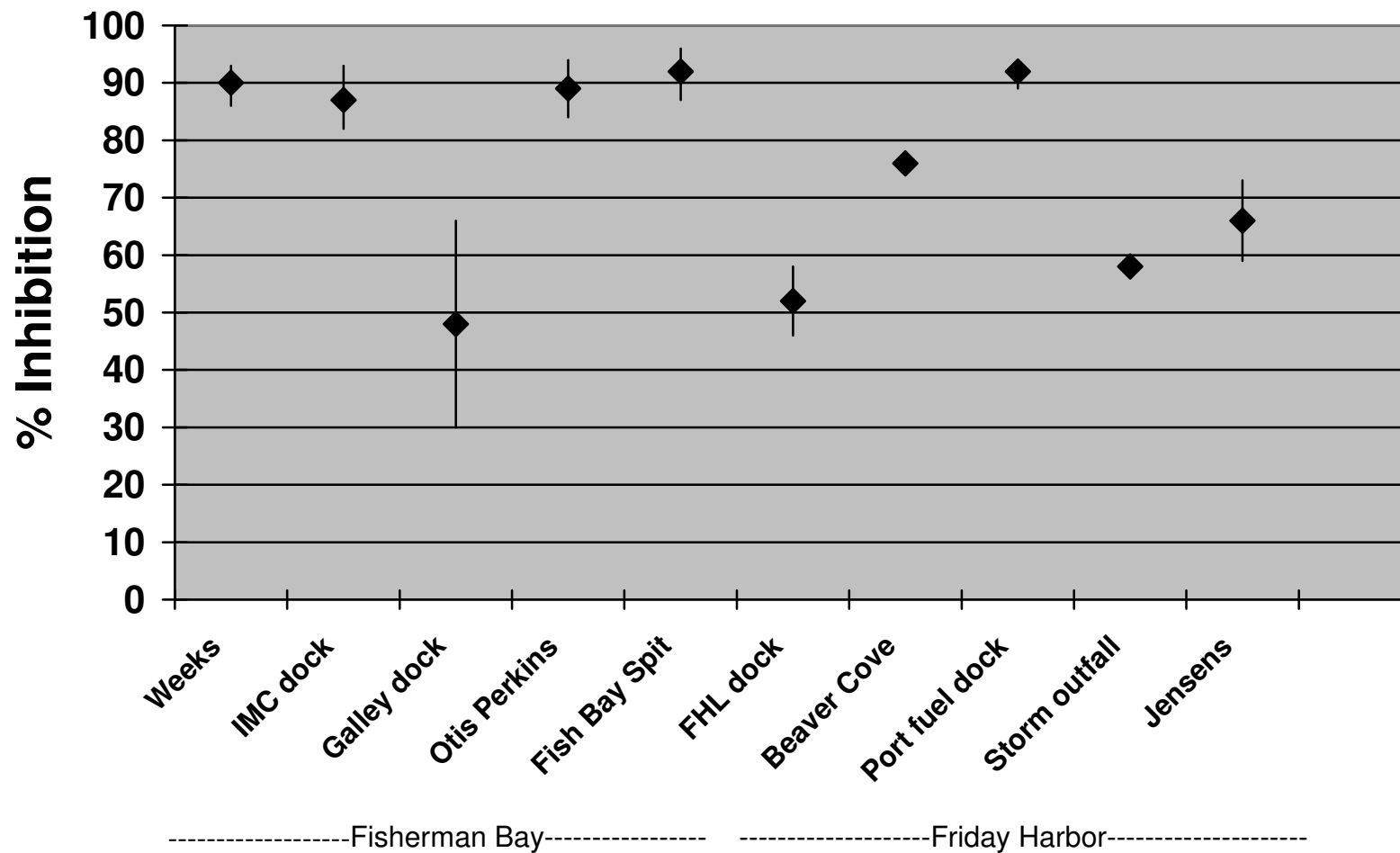


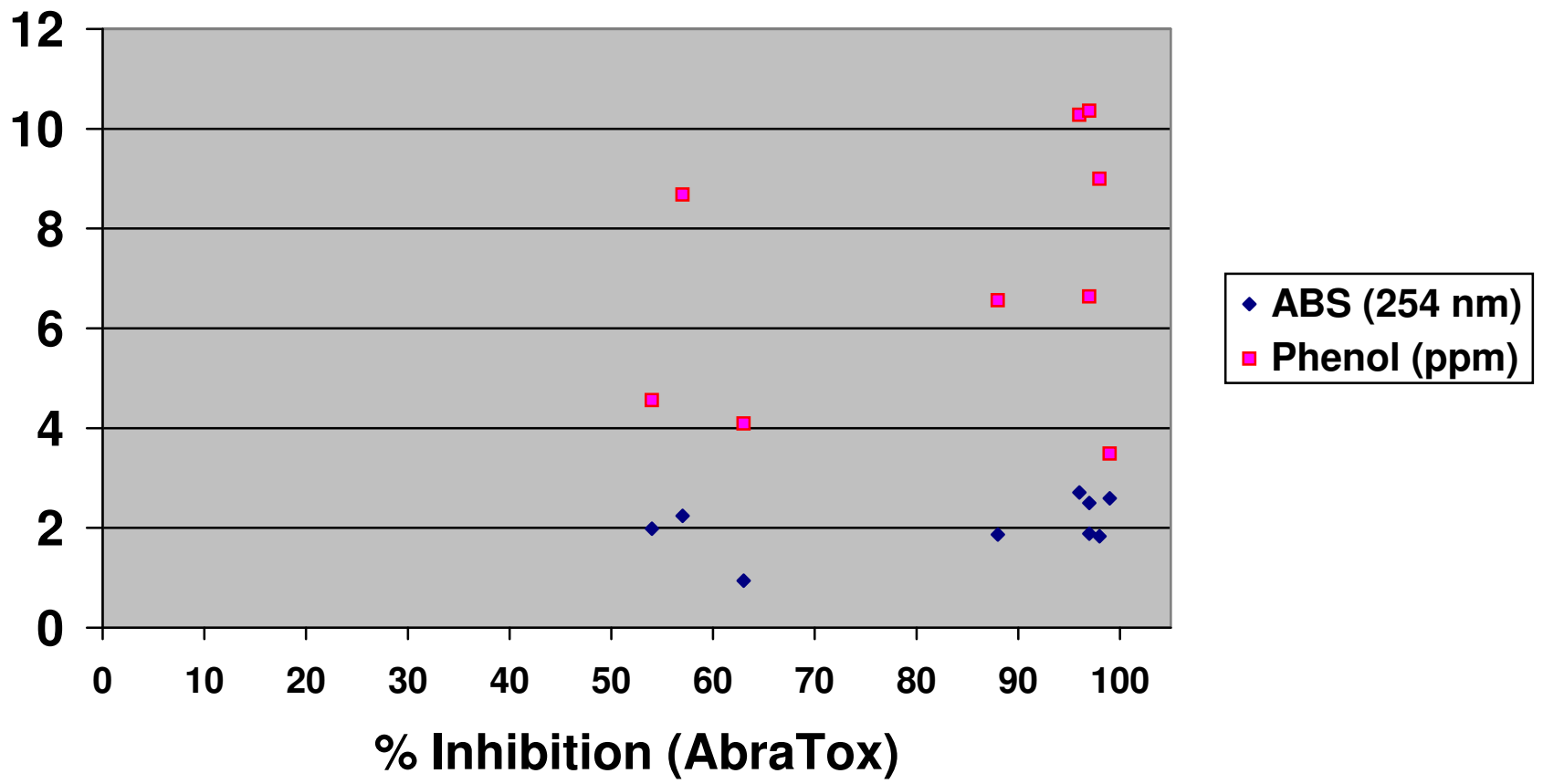
Figure IV.D. Comparison of AbraTox® results for freshwater and marine sediments from five watersheds



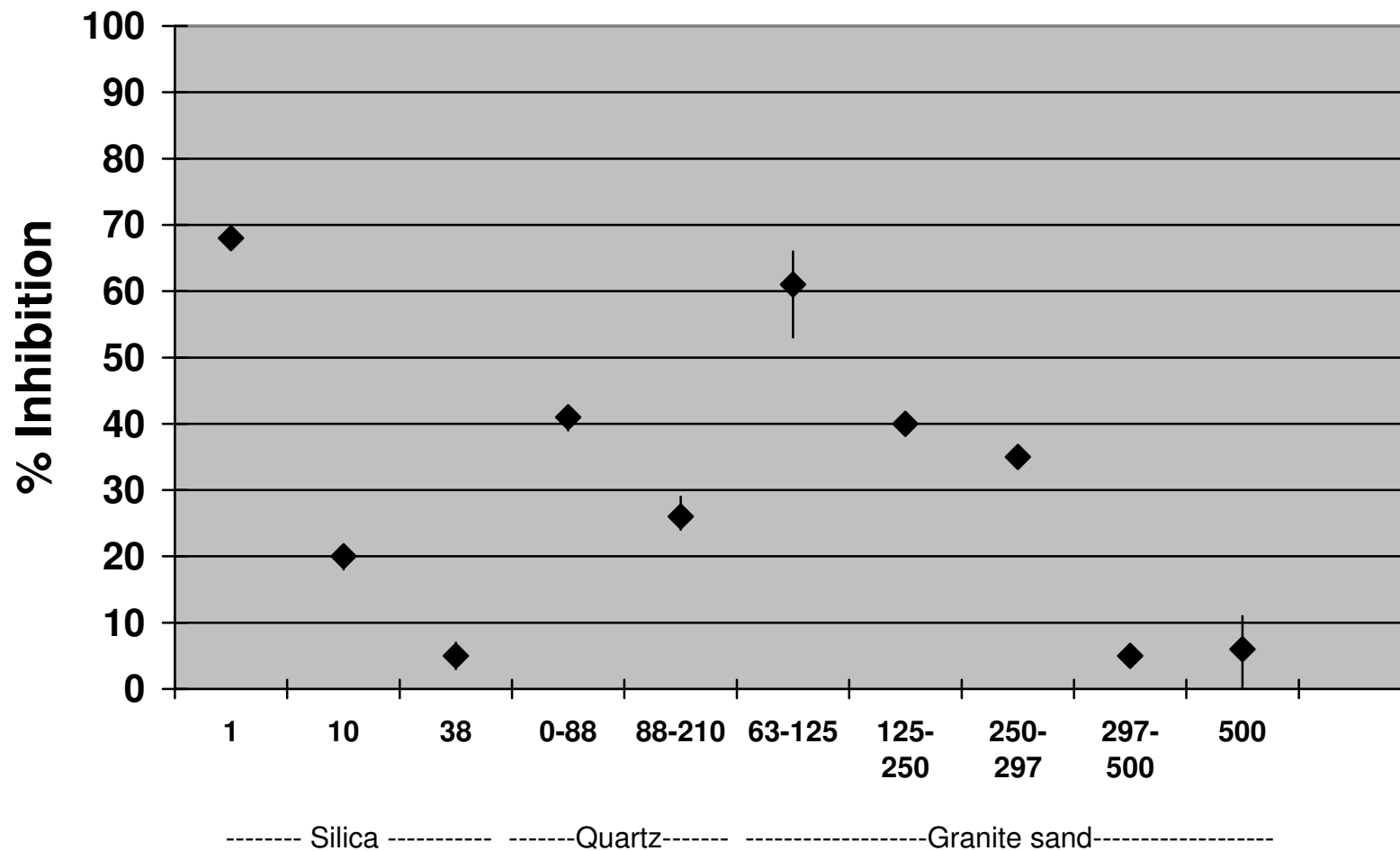
IV.E. Comparison of AbraTox® results for marine sediments in Friday Harbor and Fisherman Bay



IV.F. Comparison of AbraTox® results with other screening level data (freshwater matrices only)

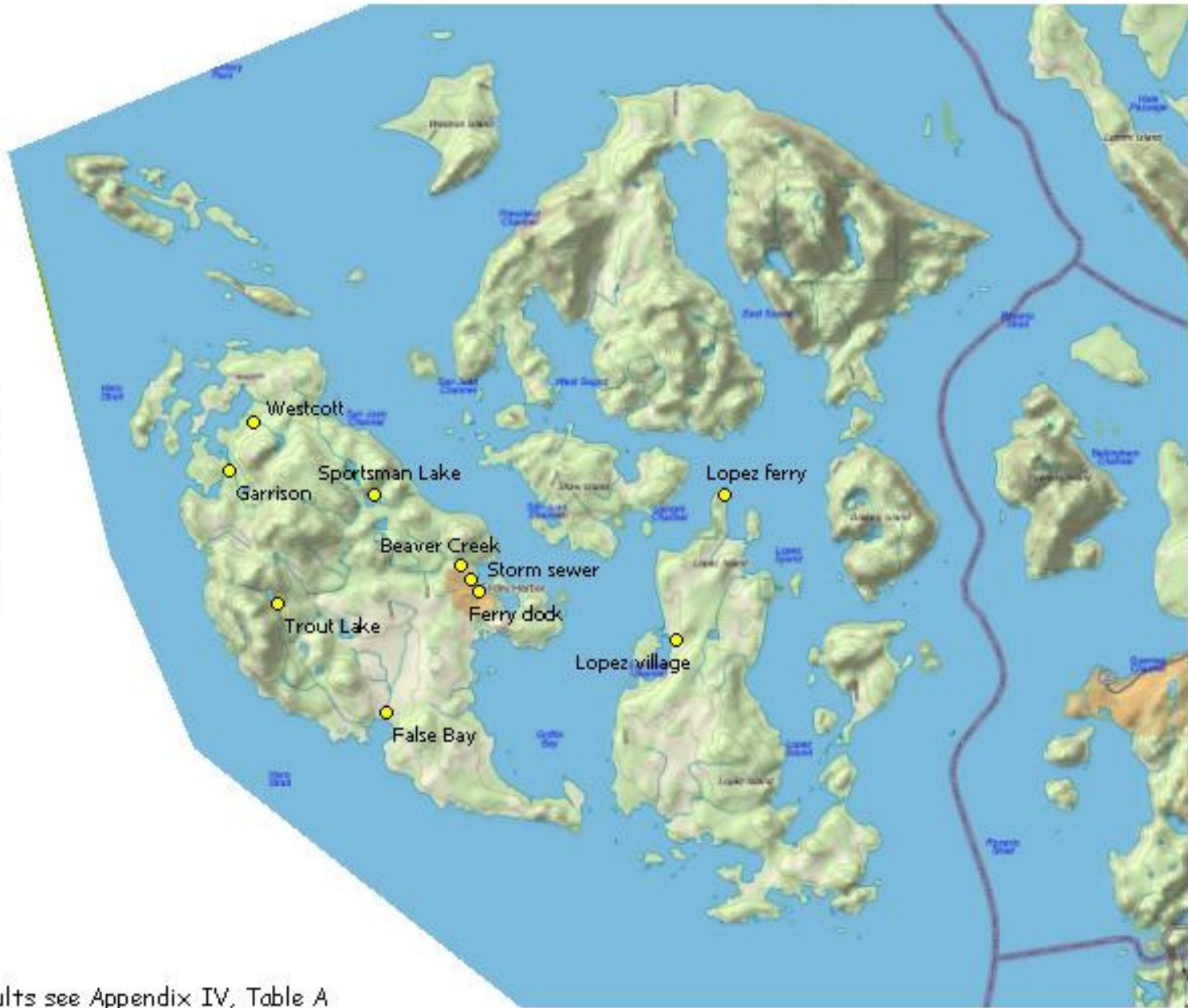


IV.F. Inhibition effect of sediment grains, by size class, on AbraTox® results





**Map 1**  
**SJC Toxicity Baseline Study**  
**Surface water sampling sites**  
**November 2008**



For results see Appendix IV, Table A





### Map 3

SJC Toxicity Baseline Study  
Sediment sampling sites  
Focus watershed  
January 2009



For results see Appendix IV, Table C



**Map 4**  
**SJC Toxicity Baseline Study**  
**Paired fresh/marine**  
**sediment sampling sites**  
**January 2009**



For results see Appendix IV, Table D



## Map 5

SJC Toxicity Baseline Study  
Inter-tidal sediment  
sampling sites  
February 2009



For results see Appendix IV, Table E

## Appendix V

### REPORTED TOXICITY to BIOLUMINESCENT BACTERIA

#### Representative toxic compounds sold in San Juan County

Compound	CAS	Source/use	LC50 ppm
1-nitronaphthalene	86-57-7	Herbicide	0.213
2,4-D	94-75-7	Herbicide	127
Biphenyl	92-52-4	Plastics	1.90
Caffeine	58-08-2	Human food stream	62.8
Carbaryl	63-25-2	Pesticide	0.636
Carbon tetrachloride	56-23-5	Dry cleaners	5.58
Chloroform (a THM)	67-66-3	Water treatment	671
Chlorpyrifos	2921-88-2	Herbicide	46.2
Cypermethrin	52315-07-8	Pesticide	8.50
Diazinon	333-41-5	Pesticide	10.3
Ethylene glycol	107-21-1	Emulsifier	621
Fenbutatin oxide	133356-08-6	Pesticide	>6
Glyphosate	1071-83-6	Herbicide	7.73
Malathion	121-75-5	Pesticide	3.01
Naphthalene (a PAH)	91-20-3	Creosote, tars, fuels	0.929
Paraquat	1910-42-5	Herbicide	777
Parathion	56-38-2	Pesticide	8.40
Permethrin	52645-53-1	Pesticide	0.566
Phenanthrene (a PAH)	85-01-8	Creosote, tars, fuels	0.073
Phenol	108-95-2	Creosote, tars, fuels	35.8
Sodium lauryl sulfate	151-21-3	Detergent	1.20
Toluene	108-88-3	Solvent	19.7
Warfarin	81-81-2	Rodent control	47.8

Source: Kaiser & Palbrica (1991).