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Nebalia pugettensis (Crustacea; leptostraca) as sediment bioassay test species

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Nebalia pugettensis (CRUSTACEA; LEPTOSTRACA) AS A SEDIMENT BIOASSAY TEST SPECIES

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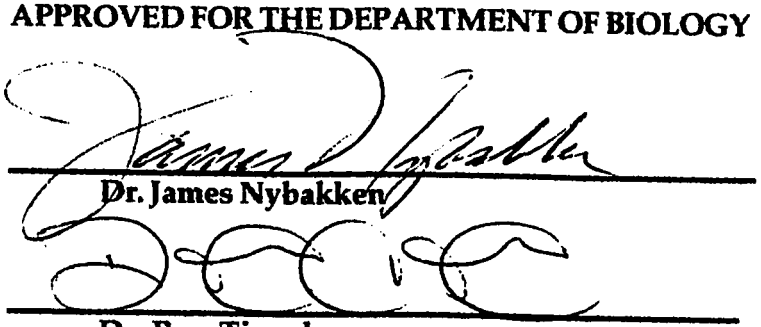

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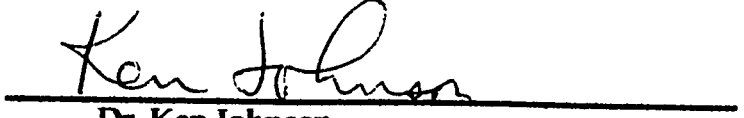

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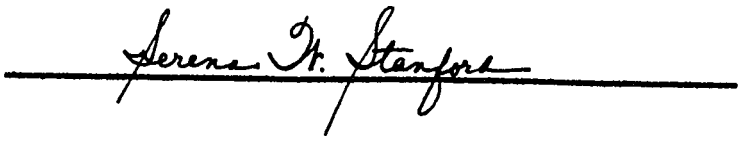

ABSTRACT

Nebalia pugettensis (CRUSTACEA; LEPTOSTRACA) AS A SEDIMENT BIOASSAY TEST SPECIES

by Kathi A. Lefebvre

Sediments in harbor and bay-type areas are frequently repositories and sources of anthropogenic contaminants. Sediments from these environments are typically fine grained and organically enriched (containing elevated levels of sulfide and ammonia). Fine grain size and organic enrichment are natural sediment characteristics which may confound the results of solid phase sediment bioassay tests. A species of leptostraca, *Nebalia pugettensis,* is commonly found in black sulfide-rich pockets of fine sediment on intertidal mud flats. Research evaluating the suitability of *N. pugettensis* as a fine grain size, sulfide and ammonia tolerant sediment bioassay test species is described here. Although *N. pugettensis,* was found to be more tolerant to natural sediment characteristics than other commonly used test amphipods, it did not appear to be sensitive to as wide of a range of anthropogenic toxins (i.e. polycyclic aromatic hydrocarbons). Additional natural history information obtained from laboratory experiments and field observations is included in Appendix A.

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INTRODUCTION

A current concern in the field of toxicology is the potential confounding effects of natural sediment characteristics on sediment bioassay test results. Many sediment bioassay protocols utilize organisms which may not be associated with the habitats being examined for toxicity. The presence of very fine grain sediments and naturally occurring ammonia and sulfides in many marine sediments are thought to confound the results of solid phase bioassay tests (DeWitt et al. 1988). Most solid phase bioassays are performed on sediments collected from areas of high organic enrichment (i.e. sewer outfalls) or protected backwater areas (i.e. harbors and bays). These habitats typically have very fine, organically rich sediments; conditions which tend to foster the development of sulfide and ammonia compounds.

Organically rich sediments may contain levels of hydrogen sulfide (H_2S) that are elevated enough to be toxic to benthic marine organisms. Production of H_2S is a natural consequence of the metabolism of organic material in anoxic sediments. The organic material can come from natural deposition of marine plant and animal remains, terrestrial runoff, and discharge of sewage effluents. If oxidation of the organic material consumes all of the oxygen dissolved in the interstitial water, then bacteria may reduce dissolved sulfate and produce H_2S. Hydrogen sulfide occurs dissolved in the interstitial water where it partially dissociates and exists in equilibrium with the hydrogen sulfide (HS^-) and sulfide ($S^=$) ions (Goldhaber and Kaplan 1973). In a Southern California Coastal Water Research Project, toxicity of H_2S was examined by reviewing studies performed with contaminated marine sediments. Sediments with the highest H_2S concentrations had the greatest

effects on test organisms, such as the white sea urchin, *Lytechinus pictus*, and the amphipod, *Grandidierella japonica* (Thompson et al. 1989, Anderson et al. 1988).

Ammonia can also be present in detrimentally high concentrations in organically rich sediments. Ammonia (NH_3 and NH_4^+) occurs naturally in aquatic ecosystems as nitrogen cycles between its organic and inorganic forms. It is acutely toxic to fish and other sensitive aquatic organisms, particularly the un-ionized form (NH_3). It has been found to be toxic to many species of marine organisms, however, few studies address the toxicity of ammonia to benthic infaunal species such as amphipods (Kohn et al. 1994).

The benthic crustaceans commonly used in solid phase tests, such as the amphipods *Rhepoxynius abronius* and *Eohaustorias estuarius*, are not naturally found in fine, organically rich sediments. They live in clean well-sorted sandy sediments (ASTM 1990). Consequently, test organisms may have poor survival when confined in these conditions. An obvious solution is the identification of a new bioassay test organism which is naturally associated with these environmental conditions and therefore inherently tolerant.

A suitable candidate is the leptostracan, *Nebalia pugettensis*, which is commonly found in pockets of black, sulfide-rich silty sediments on estuarine mud flats from Puget Sound, Washington to Baja, California (Morris et al. 1980). If *N. pugettensis* is highly tolerant to these natural sediment characteristics, yet sensitive to anthropogenic toxins, such as heavy metals, hydrocarbons and PCB's, as are other benthic crustaceans (Swartz et al. 1985), it would be an exceptional sediment bioassay test organism.

The purpose of this study was to evaluate the performance of *N. pugettensis* as a whole sediment toxicity test organism. The American Society for Testing and Materials (ASTM 1990), states that the tolerance of a test species to variations in sediment characteristics, such as particle size distribution and organic enrichment, should be established before responses can be ascribed to contaminant effects. The sensitivity of a prospective new test species should be compared with a reference species (i.e. *R. abronius*) before the new species is used in routine toxicity testing (ASTM 1990).
In order to evaluate the potential value of *N. pugettensis* as a fine grain size, sulfide and ammonia tolerant sediment bioassay test species, the following questions were addressed:

1. What are the relative tolerances of *N. pugettensis* compared to *R. abronius* to sulfide, ammonia, fine grain size, and salinity?
2. How do the relative Cd^{++} tolerances of *N. pugettensis* compare to those of *R. abronius, E. estuarius* and *Ampelisca abdita*?
3. How does *N. pugettensis* compare to *E. estuarius* and *A. abdita* in reference sediment bioassay test results?
4. How does *N. pugettensis* compare to *E. estuarius* and *A. abdita* in sediment bioassay tests using ambient contaminated sediment samples?

METHODS

Sulfide tolerance tests for *R. abronius* and *N. pugettensis* were performed at U. C. Berkeley under the direction of Dr. Susan Anderson. All other tolerance tests (ammonia, grain size, salinity and cadmium), as well as field collected sediment toxicity tests, were performed at the California Department of Fish and Game Marine Pollution Studies Laboratory. All toxicity tests followed ASTM (1990) standard procedures. Both 96-hour and ten-day exposure periods were utilized. All tests had lethal endpoints and treatment effects were measured by mean percent survival. LC_{50}'s were calculated when possible. An LC_{50} is the statistically or graphically derived best estimate of the concentration of test material added to solution or contained in sediment that is lethal to 50% of the test organisms under specified conditions within the test period. I used the trimmed Spearman-Karber method to calculate an LC_{50} and the 95% confidence limits (Hamilton et al. 1978). Appropriate water quality measurements (dissolved oxygen, salinity, temperature, ammonia, sulfide and pH) were taken for each of the tests performed. All tests were performed in 15 °C constant temperature control rooms or water baths. The number of replicates per treatment and density of organisms per test container are defined for each test in the following sections. All leptostracans utilized in this study were collected from mud flats located at the mouth of Elkhorn Slough in Moss Landing, CA (Figure 1).

Sulfide Tolerance Test Procedures

A 96-hour, closed, flow-through, exposure test was performed to determine the LC_{50} (total sulfide) for *R. abronius* and *N. pugettensis* . Tests were performed on July 13-17, 1993 and July 23-27, 1994 for amphipods and leptostracans, respectively. The design consisted of three treatments and a control, each with two replicates containing 20 animals in each replicate. Three Tedlar ® bags were filled with 1 liter Na_2S - $9H_2O$ and de-aerated deionized water solution. Each stock solution bag was connected to two jars by tubing. Precise flow was controlled by a peristaltic pump located between the bag and the jars. Each bag contained sulfide concentrations based on the desired exposure concentration for each treatment. Desired concentrations for treatments one, two and three were 2 uM, 7 uM and 15 uM, respectively, for the amphipod test and 10 uM, 50 uM and 200 uM for the leptostracan test. Bags 1, 2 and 3 contained approximately, 250 uM, 680 uM, and 1325 uM total sulfide which were corrected for 50% loss creating the desired treatment concentrations for the amphipod test. Bags 1, 2, and 3 contained approximately, 880 uM, 3400 uM and 8600 uM, respectively, creating the desired treatment concentrations for the leptostracan test. Filtered sea water was pumped from Carboys to each jar by a second peristaltic pump and tubing system. A tube containing sea water and a tube containing the sulfide solution were connected with a plastic T and then connected to a glass tube which passed through the jar lid. A second glass tube in the jar lid allowed the solution to exit the jar. This created a closed flow- through system minimizing oxidative loss of sulfide.

After 48 hours, stock solution bags were replaced with bags containing fresh sulfide solutions. The tubing containing the sulfide solution in the peristaltic pump was also replaced at day two, to prevent oxidative loss due to worn tubing. Sulfide concentrations were confirmed daily by taking samples of 10 ml from each jar, tubing junction and bag and fixing them with 10 ml SAOB (Sulfide Anti-Oxidant Buffer). Samples were read within 24 hrs with a sulfide electrode to confirm nominal concentrations. Sulfide calibration standards were made daily and a calibration curve calculated. This allowed a confirmation of the sulfide concentrations each day.

Ammonia Tolerance Test Procedures

A ten-day, water only, ammonia tolerance test was conducted concurrently with *N. pugettensis* and *R. abronius* at MPSL from January 24-February 3, 1994. Ammonium Chloride (NH_4Cl) was added to 28 psu sea water to create concentrations of 10 ppm, 20 ppm, 40 ppm, and 80 ppm for *R. abronius* and 20 ppm, 40 ppm, 80 ppm, and 120 ppm for *N. pugettensis*. For the 80 ppm and 120 ppm treatments, 43 ml and 65 ml of 74 psu brine were added to 80 ml and 120 ml ammonium chloride, respectively, and filled to one liter to correct for salinity fluctuations from 28 psu. A brine control with 43 ml and 65 ml of 74 psu brine was run for *R. abronius* and *N. pugettensis*, respectively, to account for any toxicity resulting from the brine itself. Controls containing 28 psu sea water only were also run for each test species. Ten animals were added to 500 ml of solution in closed Qorpac ® jars. Each treatment had four replicates. Solutions were renewed every 48 hours by decanting the original test solution and refilling the test container with new

test solution of the target concentration. Water quality for dissolved oxygen and salinity were taken at zero and 48 hours at each renewal. Total ammonia and pH were measured at zero, 24 and 48 hours at each renewal. Corresponding pH and total ammonia measurements were converted into unionized ammonia (NH_3^+) concentrations by the following equation (Whitfield 1974);

$$[\text{total ammonia}] \bullet (100) \bullet [1 + \text{anti Log } [pKa - pH]]^{-1}$$

Unionized ammonia concentrations were averaged in each treatment for the ten day period. An LC_{50} was calculated for both *R. abronius* and *N. pugettensis* .

Grain Size Tolerance Test Procedures

Sediment was collected at a 60 meter station located off Moss Landing Beach in Monterey Bay, California. Sediment was sieved through a series of screens to isolate grain sizes. Two grain size treatments were compared:

Coarse-- sediment passing through a .5 mm screen, but retained on a 63 micron screen. Sediment was screened three times through the 63 micron screen to make sure all fine sediment had been rinsed out.

Fine-- sediment passing through a 63 micron screen.

The two treatments, coarse and fine sediment, each had five replicates containing 20 leptostracans each. One liter beakers were filled with sediment to a depth of 2 cm and with sea water to one liter. This test was only performed with *N. pugettensis*. A t-Test (p=.05) was used to compare mean

percent survivals of coarse and fine treatments. Grain size analysis was performed on a sample of the fine test sediment to determine percent fines.

Salinity Tolerance Test Procedures

On July 19, 1994, test animals were brought to MPSL and placed in an aquarium containing ambient (33 psu) sea water. On July 20, 1994, 80 leptostracans were moved from the original tank (33 psu) to a tank containing 28 psu sea water. Likewise, on July 21, 1994, the leptostracans in the 28 psu sea water were moved to 23 psu sea water and 80 leptostracans from the 33 psu water were moved to the 28 psu tank. This was repeated until 80 leptostracans were separated into each of the five types of sea water, 33, 28, 23 and 18 psu. All animals remained in their final test salinity treatment for 24 hours before being placed into test beakers filled with appropriate sea water. Each treatment was replicated three times and contained twenty leptostracans per beaker. Mean percent survivals in each of the five treatments were compared using a one-way ANOVA (p=.05). An LC_{50} was calculated.

Cadmium Tolerance Test Procedures

The relative sensitivity of *N. pugettensis* and *R. abronius* to $CdCl_2$ was compared in concurrent, 96-hour, water only exposures. Cadmium chloride (0.1630 g) was mixed with one liter of distilled water to create a 100 mg/l stock solution. One liter of sea water with no Cd^{++} was used for a control treatment. Sensitivity to four concentrations was compared: 0.50, 1.00, 2.00 and 4.00 mg/l. Each treatment was replicated five times. Three tests were conducted at 28 psu for *N. pugettensis*. Three additional tests were conducted

at 33 psu (ambient) for *N. pugettensis*. This procedure was repeated for *R. abronius, A. abdita* and *E. estuarius* at 28 psu only. A one-way ANOVA (p=.05) was used to compare mean percent survivals between treatments. An LC_{50} was calculated.

Cadmium chloride was also used as a reference toxicant. 96-hour $CdCl_2$ tests were performed concurrently with each field collected sediment toxicity test for each test species. Reference toxicant tests are used to monitor the consistency of the test species toxicity response, laboratory performance, temporal variability and overall health of the test organism. Standard sensitivity ranges have been determined for many commonly used test species. Reference toxicant test results must fall within these ranges in order for results of the accompanying sediment test to be considered valid.

Field Collected Sediment Toxicity Tests

Mean percent survivals of *N. pugettensis* in field collected sediment toxicity tests using both reference (uncontaminated) and toxic sediments were used to evaluate the performance of *N. pugettensis* compared to other regulation test amphipods. Whenever possible, *E. estuarius* and *A. abdita* were tested concurrently with *N. pugettensis* in the same sediments. These tests were also used to determine relative sensitivity of *N. pugettensis* to anthropogenic toxins. Test sediment was collected from field stations in San Francisco Bay chosen by the San Francisco Regional Water Quality Control Board (SFRWQCB) for use in a reference site identification project. The two-year SFRWQCB project, was designed to identify better in-Bay reference sites and more realistic toxicity tests for use by Bay dredgers, dischargers, toxic

clean-up planners and regulators (Estuary 1995). Results from bioassay tests in potential reference sediment treatments were compared to results from tests in sediments from known toxic hot spots.

For the present study, *N. pugettensis* was tested in sediment samples from 21 field stations in San Francisco Bay. Sixteen of the 21 sites tested with leptostracans were "clean" reference sites. Five additional sites, specifically chosen because they are known to be toxic, were tested for comparison to reference sediment test results. Field stations were sampled at various times throughout the study. Separate sampling dates are distinguished by Leg numbers. Leg 31 sediment was collected from five sites in May of 1994. Leg 35 sediment was collected from six sites in September of 1994. Leg 37 sediment was collected from seven sites in April of 1995. Leg 38 sediment was collected from three sites in June of 1995. Ten day sediment tests with five lab replicates from each of the field stations, along with five home sediment replicates as controls were performed with three test species (*N. pugettensis*, *E. estuarius*, and *A. abdita*) on May 6, 1994, September 20, 1994 and April 7, 1995, for legs 31, 35 and 37a, respectively. Two additional sites from leg 37 (called 37b) were tested later on April 17, 1995. In the final test of field sediment, leg 38, only *N. pugettensis* was tested in sediment from all three field stations: East China Basin, Guadalupe Slough and Lauritzen Channel. *E. estuarius* was tested in sediment collected on an earlier sampling trip from two of the sites: East China Basin and Guadalupe Slough.

In each test, twenty animals were placed in one liter test containers with 3 cm of sediment and sea water filled to one liter. For example, 20 leptostracans were placed in each of the five lab replicates from each field

station and five home sediment control containers. This was repeated for *E. estuarius*, and *A. abdita* . Tests with all three species were started within seven days of each other. Sea water of 28 psu was used in all tests with *E. estuarius*, and *A. abdita*. Sea water of 28 psu was used in Leg 31 for *N. pugettensis*. Ambient sea water of 33 psu was used in Leg 35, 37 and 38 for *N. pugettensis*. A Cd^{++} reference toxicant test with each test species was started the same day as the sediment tests and taken down after 96 hours. Methods for the reference toxicant test are identical to those described previously for the Cd^{++} tolerance procedure.

Survival of test organisms in each field station sediment were compared to the home sediment control treatment with t-tests (p= .05). These results were compared between test species. For the Cd^{++} reference toxicant test, a one-way ANOVA was used to compare mean percent survivals between treatments. An LC_{50} was calculated and compared to previously calculated LC_{50}'s.

Feeding Protocol Tests

Based on poor survival results in all of Leg 31 reference sediment treatments, feeding protocol tests were performed to see if feeding was required during ten day sediment toxicity tests. In feeding protocol test one, performed June 13, 1994, leptostracans from the same stock as those utilized in Leg 31 were tested. This stock was originally collected from the field on July 19, 1993. The leptostracans had been in culture in laboratory aquaria for over 11 months when tested in Leg 31 reference sediments and in the first feeding protocol test. Three treatments and a control were replicated three

times. Each one liter test beaker contained 3 cm of clean sediment, ambient (33 psu) sea water and 20 leptostracans. Eight mg of Tetramin ® flake fish food per beaker were added once, twice and four times throughout the ten day test for treatments one, two and three, respectively. Sea water was renewed with each feeding by decanting the original test solution and adding fresh sea water. The control treatment consisted of one sea water renewal at 24 hours and no feeding. Water quality measurements were taken at the start and end of each renewal.

Feeding protocol test two was performed on July 2, 1994 with leptostracans freshly collected from the field on June 29, 1994. Three treatments and two control treatments were replicated three times. Four mg and 2 mg of Tetramin ® flake fish food were added on day one to treatments one and two, respectively. For treatment three, 2 mg of Tetramin were added on day one and day five. In all three treatments, sea water was renewed on day five. To rule out the possibility of deleterious effects due to over crowding, an extra control treatment containing ten leptostracans per replicate, instead of 20, was added. Leptostracan survival was compared between treatments with a one way ANOVA ($p = .05$).

RESULTS

Unless otherwise stated, water quality measurements for all experiments fell within acceptable standard ranges established by MPSL.

Sulfide Tolerance

Sulfide concentrations are extremely difficult to keep stable because of fast oxidation rates. A closed flow-through system was required to maintain relatively constant sulfide concentrations. Daily sulfide concentrations in the *N. pugettensis* test fluctuated from 15 to 2 µM, 59 to 24 µM and 148 to 119 µM in treatments one, two and three, respectively. Daily sulfide concentrations in the test utilizing *R. abronius*, fluctuated from 2 to 0 µM, 10 to 1 µM and 26 to 2 µM in treatments one, two and three, respectively. Although sulfide concentrations fluctuated radically, especially in the amphipod test, the results indicate that *N. pugettensis* is several times more tolerant to sulfide than *R. abronius* (Figure 2). Because the lowest survival for *N. pugettensis* was 75 %, an LC_{25} was calculated instead of an LC_{50}. *N. pugettensis* had an LC_{25} of 111.06 µM total sulfide. *R. abronius* had an LC_{50} of 9.73 µM total sulfide.

Ammonia Tolerance

An LC_{50} of 0.44 mg NH_3/l, with 95 % confidence limits of 0.40 - 0.48 mg NH_3/l, unionized ammonia was calculated for *R. abronius*. An LC_{50} of 0.74 mg NH_3/l, with 95 % confidence limits of 0.70 - 0.78 mg NH_3/l, was calculated for *N. pugettensis*. These numbers indicate a slightly higher tolerance by *N. pugettensis* to ammonia. Percent survival was 62.5 % for *N.*

pugettensis in both brine and regular controls. Percent survival for *R. abronius*, was 62.5 % in the sea water only control and 65 % in the brine control. Because this was a ten day test in water only, these control survival rates were acceptable. Absence of sediment appears to add stress and reduce the % survival of amphipods in ten day tests.

Grain Size Tolerance

N. pugettensis demonstrated no significant difference in survival between coarse and fine sediment treatments with 94% and 97% survival in each treatment, respectively. Through sieve and hydrometer analysis, the fine sediment treatment was shown to contain 98.88 % total fines (% silt + % clay) and 1.12 % medium/fine sand (Table 1).

Salinity Tolerance

N. pugettensis had no statistically significant difference in survival between the 23 psu, 28 psu and 33 psu treatments with mean percent survivals of 58 %, 76 % and 86 %, respectively. There was a significant difference ($p = .05$) between these treatments and the 13 psu and 18 psu treatments in which there was 0 % survival (Figure 3). These results show a trend of decreasing survival with lower salinity.

Cadmium Tolerance

Cadmium LC_{50}'s for *A. abdita, R. abronius, N. pugettensis*, and *E. estuarius* obtained during this study, are listed in Table 2a. Standard Cd^{++} LC_{50} ranges from 96-hour, water-only exposures taken from ASTM (1990), are

listed in Table 2b. *N. pugettensis* LC_{50}'s are well within the range of sensitivities encompassed by the commonly used amphipod species.

Field Collected Sediment Bioassay Tests; Leg 31, 35, 37a ,37 b and 38

Results of reference sediment test Leg 31 with *N. pugettensis, A. abdita* and *E. estuarius* are shown in figure 4. Leptostracans utilized in this test had been cultured in laboratory aquaria for over 11 months prior to testing. Mean percent survival of *N. pugettensis* was poor in all treatments. Mean percent survival in the control was 18 %, constituting a failed test. The highest mean percent survival was 69 %. In all other treatments, mean percent survival was below 50 % (Figure 4). Mean percent survival was above 73 % in all sites and 80 % in the control for *A. abdita* in Leg 31 (Figure 4). The lowest mean percent survival for *E. estuarius* was 32 %. In all other treatments, mean percent survival was above 71 % with a control survival of 93 % for *E. estuarius* (Figure 4). An LC_{50} of 1.20 mg/liter was obtained from the Cd^{++} reference toxicant test for *N. pugettensis* (Table 2a). This was the second lowest LC_{50} calculated throughout this study for *N. pugettensis*. Prolonged laboratory holding time may be responsible for the poor control survival and higher sensitivity to cadmium observed in this test. Affects of prolonged laboratory holding time on test organism performance are discussed further in the following feeding protocol section.

The leptostracans utilized in Leg 35 were collected from the field on June 29, 1995, and were held in the laboratory for approximately two months prior to testing. Percent survival for *N. pugettensis* was above 90% for all six sites and the control treatment (Figure 5). Sediments from Castro Cove and

Islais Creek caused significant mortality for both *E. estuarius* and *A. abdita* compared to controls (Table 5). *N. pugettensis* was not affected by the toxic sites (Table 5). An LC_{50} of 1.72 mg/l was calculated from the Cd^{++} reference toxicant test (Table 2a).

Reference Sediment Test Leg 37a, was performed with leptostracans that had been cultured in laboratory aquaria for approximately six months. Mean percent survival was 85 % in the control and ranged from 65 to 70 % in all other treatments for *N. pugettensis* (Figure 6). Control survival was above 85 % for both *E. estuarius* and *A. abdita*. Mean percent survival ranged from 57 to 82 % and from 82 to 97 % in all other sites for *E. estuarius* and *A. abdita*, respectively (Figure 6). An LC_{50} of 1.58 mg/liter was calculated from the Cd^{++} reference toxicant test for *N. pugettensis* (Table 2a).

Because leptostracans were not available in the field, organisms from a nine month old culture stock had to be used in Leg 37b. Control survival was 19 % for *N. pugettensis*, constituting a failed test (Figure 7). Mean percent survival was 54 and 40 % in the other sites (Figure 7). Control survival was above 90 % for both *E. estuarius* and *A. abdita* (Figure 7). Mean percent survival was 80 and 85% for *E. estuarius*, and 79 and 89% for *A. abdita* in both sites of Leg 37b (Figure 7). An LC_{50} of 0.80 mg/liter was obtained from the Cd^{++} reference toxicant test for *N. pugettensis* (Table 2a). This was the lowest LC_{50} calculated throughout this study for *N. pugettensis*.

The two most toxic sites identified from SFRWQCB's reference site screening project were chosen for Leg 38. These sites included East China Basin and Guadalupe Slough. Lauritzen Channel was additionally chosen for Leg 38 because of its known toxicity. This site has been well characterized

chemically and toxicologically. Leptostracans utilized in Leg 38 were freshly collected from the field and held less than one week in laboratory aquaria before testing. Control survival was 98 % for *N. pugettensis*. Mean percent survival was 96, 94 and 51% for East China Basin, Guadalupe Slough and Lauritzen Channel sediments, respectively (Table 3). *N. pugettensis* had significant mortality in the Lauritzen Channel sample, but not in the two other samples (Table 3). Control survival was 99% for *E. estuarius*. *Eohaustorius estuarius* had significant mortality in the East China Basin sample, but not in Guadalupe Slough sediment (Table 3). Unfortunately, *E. estuarius* was not tested in the Lauritzen Channel sediments because this site was not part of the original reference site screening program.

A comparison of control survival rates of leptostracans held in the laboratory for two, six, nine and 11 months, reveal a trend of poorer survival with longer holding times (Figure 8). Cd^{++} LC_{50}'s with leptostracans held for the same time periods of two, six, nine and 11 months, exhibit a similar trend in sensitivity with LC_{50}'s of 1.72, 1.58, 0.80 and 1.20 mg/l, respectively. *N. pugettensis* appears to be more sensitive in sediment tests and reference toxicant tests with prolonged laboratory holding times.

Feeding Protocol Tests

Percent survival was below 50 % for all treatments in the first feeding protocol test (Table 4). Leptostracans utilized in this test had been cultured in laboratory aquaria for over 11 months. There were no significant differences in survival between feeding treatments and the control.

In the second feeding protocol test, utilizing leptostracans freshly collected from the field, percent survival was above 95% for all treatments (Table 4). There were no significant differences in survival between the feeding treatments, control (containing 20 leptostracans per replicate) and control (containing 10 leptostracans per replicate). Feeding apparently had no effect on survival rates in ten day tests. The leptostracans held for long periods in the laboratory had significantly lower survival rates in the first feeding protocol test than freshly collected organisms utilized in the second feeding protocol test (Table 4).

DISCUSSION

The goal of this project was to evaluate the suitability of the leptostracan, *Nebalia pugettensis*, as a sediment toxicity test species. Desirable properties for an appropriate sediment toxicity test species, suggested by DeWitt et al. (1989), are: (1) occupation of microhabitats at, or preferably, below the sediment-water interface to ensure maximum and consistent exposure to sediment contaminants; (2) broad geographic range to enhance the breadth of its application as a test species; (3) ease of collection, handling and maintenance in the laboratory; (4) high survival under control conditions; (5) the ability to be cultured or year-round availability from the field; (6) low sensitivity to natural sediment variables, such as organic content and particle size, to allow a wide variety of sediment types to be tested; and (7) high sensitivity to common sediment contaminants. The following discussion will address the performance of *N. pugettensis* in each of these categories.

Habitat, Geographic Range and Ease of Collection

The natural habitat, geographic range, and ease of collection of *N. pugettensis* meet the criteria suggested for an ideal sediment toxicity test species. *N. pugettensis* is an infaunal burrower which brings it into almost constant contact with sediment particulates and interstitial water. This ensures maximum exposure to sediment-bound contaminants without interference or dilution from water overlying the sediment. It is broadly distributed along the North American Pacific coast from Puget Sound,

Washington to Baja, California. *N. pugettensis* was rapidly and inexpensively collected in its intertidal mud flat habitat. In several collection efforts, I was able to collect over 1,500 leptostracans in less than two hours with a petri dish and several zip lock ® bags.

Control Survival

N. pugettensis had high control survival in all experiments performed with freshly collected leptostracans. In clean sediments from Moss Landing beach, California, utilized in Feeding Protocol Test Two, mean percent survival was above 96% in all treatments (Table 4). Mean percent survival was 100 % for controls in both Leg 35 and Leg 38 (Figure 5). However, control survival was poor in tests performed with leptostracans held longer than six months in laboratory aquaria (Figure 8).

Availability

On the mud flats sampled in this study, leptostracans were not available in the field year round (see Appendix A). At first appearance, *N. pugettensis* could be easily cultured in laboratory aquaria. Prolific populations were sustained throughout the two year period in the laboratory from stock organisms collected from the field. However, leptostracans held longer than six months in laboratory aquaria had lower survival in all treatments and controls when utilized in any type of ten-day test, than organisms utilized within two months of collection from the field (Figure 8). Cd^{++} sensitivity in 96-hour exposures also increased slightly with longer laboratory holding times. The two lowest Cd^{++} LC_{50}'s (1.20 and 0.80 mg/l)

calculated were with leptostracans taken from a culture stock over nine months old (Table 2a). It appears that something is missing (perhaps a diet item) in the laboratory aquaria habitat that is available in the field. Appropriate laboratory cultures, identical to natural field conditions, may be possible by using sediment collected from the natural mud flat habitat for laboratory aquaria cultures, instead of the clean well-sorted sand used in this study. *R. abronius* and *E. estuarius* have also been shown to have increased sensitivity to various toxicants with prolonged laboratory holding time (Meador 1993). Meador (1993) found that amphipods held for several weeks in the laboratory before tests were 2-3 times more sensitive to Cd^{++} and Tributyltin than organisms held for only a few days prior to testing.

Sensitivity to Natural Sediment Variables

N. pugettensis had a lower sensitivity to some natural sediment variables compared to other commonly used sediment toxicity test amphipods. As previously mentioned, sediments being examined for toxicity are typically collected from areas of high organic enrichment. Possible confounding effects caused by natural sediment characteristics (elevated sulfide and ammonia levels, and fine grain size) have become a concern in toxicity tests performed with benthic amphipods. *N. pugettensis* appears to be highly tolerant to sulfide, especially when compared to *R. abronius* (Figure 2). The LC_{25} of 111.06 µM for *N. pugettensis* is several times higher than the LC_{50} of 9.73 µM calculated for *R. abronius* in 96-hour water-only sulfide tests. This is not surprising since *N. pugettensis* is commonly found in pockets of black sulfide-rich sediment, while *R. abronius* is characteristically found in

well-sorted sand. Additional LC_{50}'s of 50 and 104 µM total sulfide were calculated for *R. abronius* and *E. estuarius*, respectively, from 48-hour water-only exposures (Knezovich and Gelinsky, U. C. Berkeley Marine Laboratory, personal comm.). These are somewhat higher, but higher tolerances are expected in shorter exposure periods (48-hours versus 96-hours). *N. pugettensis* is considerably more tolerant to sulfide than either *R. abronius* or *E. estuarius*.

Nebalia pugettensis appears to be slightly more tolerant to ammonia than *R. abronius*, when tested under static water-only conditions for ten days. Treatments containing *R. abronius* and *N. pugettensis* were run concurrently. The absence of sediment stressed both test species, exemplified by survival rates of 62 % in controls, and may have increased their sensitivity to ammonia. LC_{50}'s of un-ionized ammonia were 0.44 mg/l for *R. abronius* and 0.74 mg/l for *N. pugettensis*. In a study by Kohn et al. (1994), LC_{50}'s of 1.59, 0.83 and 2.49 mg/l un-ionized ammonia were calculated from 96-hour water-only exposures for *R. abronius*, *A. abdita* and *E. estuarius*, respectively. The LC_{50} of 0.44 mg/l for *R. abronius* calculated in this study, is lower than the LC_{50} of 1.59 mg/l reported by Kohn et al. (1994); however, higher sensitivities are expected in ten-day exposures than in 96-hour exposure periods.

The third natural sediment variable of recent concern, is sediment grain size. *N. pugettensis* showed no sensitivity to sediments of different grain sizes: mean survival was 97 % in the fine sediment treatment and 94 % in the coarse sediment treatment. The fine sediment treatment consisted of 98.88 % fines (Table 1). Although *R. abronius* was not tested in this sediment

test, a previous study by DeWitt et al. (1988) revealed that amphipod survival in fine, uncontaminated, field sediments (≥ 80 % silt-clay) were up to 15 % lower than survival in native sediments. Survival of *R. abronius* was affected by sediment particle size. As the % Fines (i.e. the silt-clay fraction) content of a sediment increased, amphipod survival decreased (DeWitt et al. 1988). In a more recent study by DeWitt et al. (1989), sensitivity to sediment particle size was compared between *E. estuarius* and *R. abronius*. In this case, mean survival declined slightly for both species as grain size decreased. However, correlations between survival and grain size were uniformly low, indicating little effect on either amphipod (DeWitt et al. 1989). *R. abronius* showed greater tolerance to fine sediments in the 1989 study than reported previously by DeWitt et al. 1988. The cause of this difference in sensitivity to fine sediments is not known, nor is there a consensus on the ultimate cause of mortality of these species in some fine, uncontaminated sediments (DeWitt et al. 1989).

Sensitivity to Common Sediment Contaminants

Cd^{++} reference toxicant tests were used to determine the sensitivity of *N. pugettensis* to a common anthropogenic sediment contaminant. Cadmium toxicity was chosen as an indicator of leptostracan sensitivity to anthropogenic toxins because of its widespread use and acceptance as an appropriate reference toxicant (ASTM 1990). A total of six 96-hour sea water-only exposures with $CdCl_2$ were performed. LC_{50}'s from these reference toxicant tests fell well within the range of sensitivities of other commonly used sediment toxicity test species. *N. pugettensis* was slightly more tolerant

to Cd^{++} than *A. abdita* and *R. abronius*, yet more sensitive than *E. estuarius* (Table 2).

A broad range of whole sediment toxicity tests were another measurement used to determine the sensitivity of *N. pugettensis* to common sediment contaminants. Of 21 samples collected for this study, five showed significant toxicity with at least one species tested. These sites were, Castro Cove and Islais Creek from Leg 35 and East China Basin, Guadalupe Slough and Lauritzen Channel from Leg 38. The amphipods *E. estuarius* and *A. abdita* exhibited acute toxicity responses in sediments collected from Castro Cove and Islais Creek, while *N. pugettensis* had 100 % and 97 % survival, respectively (Table 3). Castro Cove is an area highly contaminated with Polycyclic Aromatic Hydrocarbons (PAH's). Up until 1972, Chevron Oil Corporation had been discharging untreated oil refinery effluent into Castro Cove (Karen Taberski, San Francisco Regional Water Quality Control Board, personal comm.). As a result, sediments from this area appear tar-like and are extremely toxic to most organisms tested in it. *N. pugettensis* was not affected by the contaminants found in this sediment.

Islais Creek is a site influenced by sewage outfalls from San Francisco. Several overflow pipes feed into the area allowing untreated sewage to flow into the canal when rains are heavy. Unfortunately, the source of toxicity to the organisms tested in this sediment could not be clearly identified. Further analysis of the Islais Creek sediments, in which urchin larvae where utilized in a series of Toxicity Identification Evaluations (TIE's), were performed. TIE's are a series of tests designed to characterize the physical/chemical nature of the constituents which cause toxicity and specifically identify toxicants if

they are non-polar organics, ammonia, or metals. Unfortunately these evaluations are incomplete because methods for other specific toxicant groups, such as polar organics, have not yet been developed (MATIE 1991). As a result, polar organics were suggested as the source of toxicity in Islais Creek sediments through the process of elimination (Karen Taberski, SFRWQCB, personal comm.). Hydrogen Sulfide also appeared to be the cause of some toxicity. Again, *N. pugettensis* was not affected by contaminants in Islais Creek sediments.

A third site, East China Basin, exhibited extreme toxicity with *E. estuarius,* while *N. pugettensis* was again unaffected (Table 3). East China Basin receives storm drain runoff directly from the streets of San Francisco. Typical contaminants contained in this type of runoff are metals and PAH's (oil and grease). In addition, this site receives untreated sewage from overflow pipes when rains are heavy. Water storage areas have not been sufficient to stop raw sewage from flowing into East China Basin due to flooding (Karen Taberski, SFRWQCB, personal comm.).

In the fourth presumably toxic site, Guadalupe Slough, both *N. pugettensis* and *E. estuarius* were not significantly affected (Table 3). However, this site was chosen because toxicity tests with urchins in pore water from these sediments, did show significant mortality (Table 3). Guadalupe Slough is located in the South Bay and receives sewage discharge that is highly treated. As with Islais Creek, further testing, utilizing TIE's performed with urchin larvae, suggested polar organics to be the cause of toxicity.

The final toxic site, Lauritzen Channel, was chosen because of its designation by the U. S. Environmental Protection Agency (EPA) as a Superfund site due to historic contamination (U. S. EPA 1990). Property adjacent to the Lauritzen Channel was used by various corporations to formulate and grind DDT and dieldrin from approximately 1945 to 1966 (Levine-Frick 1990). This activity resulted in substantial contamination of the soils and sediments. Remedial action in 1990 removed the worst contamination from the embankment of the Lauritzen Channel, where in some areas the DDT concentration in the soil was virtually 100 %. Despite this action, the sediments in the area remained contaminated by DDT and dieldrin. The objective of a study by Swartz et al. (1994) was to evaluate the relative contribution of DDT, dieldrin, PAHs, PCBs and metals to sediment toxicity and effects on amphipod abundance in the Lauritzen Channel. Experimental and corroborative field data, accumulated in Swartz's study, indicated that DDT was the major factor causing sediment toxicity and a depression in amphipod populations in the Lauritzen Channel. First, the metal-to-AVS (Acid-volatile sulfide) molar ratio and the TUs (toxic units) of dieldrin, PCB (Aroclor 1254), and PAH were too low, whereas the TUs of DDT were sufficient to exert acute toxicity. Second, the patterns of DDT concentration response (sediment toxicity and amphipod abundance), the thresholds of response, and the field-derived LC_{50}'s for DDT were similar at three sites of DDT contamination. Together, these data provide strong evidence that DDT is the dominant ecotoxicological factor in the Lauritzen Channel (Swartz et al. 1994). *N. pugettensis* exhibited an acute toxicity response when tested in sediments from this area (Table 3). Although

Nebalia appears to be highly tolerant in contaminated sediments from the first four toxic sites described, it appears to be sensitive to the DDT dominated sediments of Lauritzen Channel.

Conclusions

According to many of the desirable properties for an appropriate sediment bioassay test species listed above, *N. pugettensis* appears to be a prime candidate. It is an infaunal burrower in constant contact with sediment particulates and interstitial water, is broadly distributed, has high survival in control treatments with freshly collected organisms, is easily and inexpensively collected, may be cultured (with further experimentation), is tolerant of natural sediment variables and exhibits similar sensitivities in Cd^{++} reference toxicant tests as other commonly used sediment toxicity test species. However, in actual solid phase toxicity tests with contaminated sediments, *N. pugettensis* appears to be less sensitive than other currently used amphipod test species, such as *A. abdita* and *E. estuarius*.

In the first four toxic sites described; Castro Cove, Islais Creek, East China Basin and Guadalupe Slough *N. pugettensis* gave no indication of toxicity, while other test species were significantly affected. The leptostracans utilized in all of these sediments were freshly collected from the field and therefore presumably revealed realistic natural tolerances. Unfortunately, the exact source of toxicity could not be determined for Islais Creek, East China Basin, and Guadalupe Slough with TIE analysis utilizing urchins and amphipods. However, the source of toxicity for two of the sites tested can be realistically defined as PAH's for Castro Cove and DDT for Lauritzen

Channel. *N. pugettensis* appeared to be completely unaffected by the PAH contaminated sediment, yet was significantly affected by the DDT contaminated sediment. These results leave an unclear answer as to the usefulness of *N. pugettensis* for sediment bioassay testing. This species does not appear to be sensitive to as wide of a range of contaminants as other currently used test species. However, because of its tolerance to sulfide, comparable sensitivity to Cd^{++}, and sensitivity to DDT, *N. pugettensis* may be useful in specific cases.

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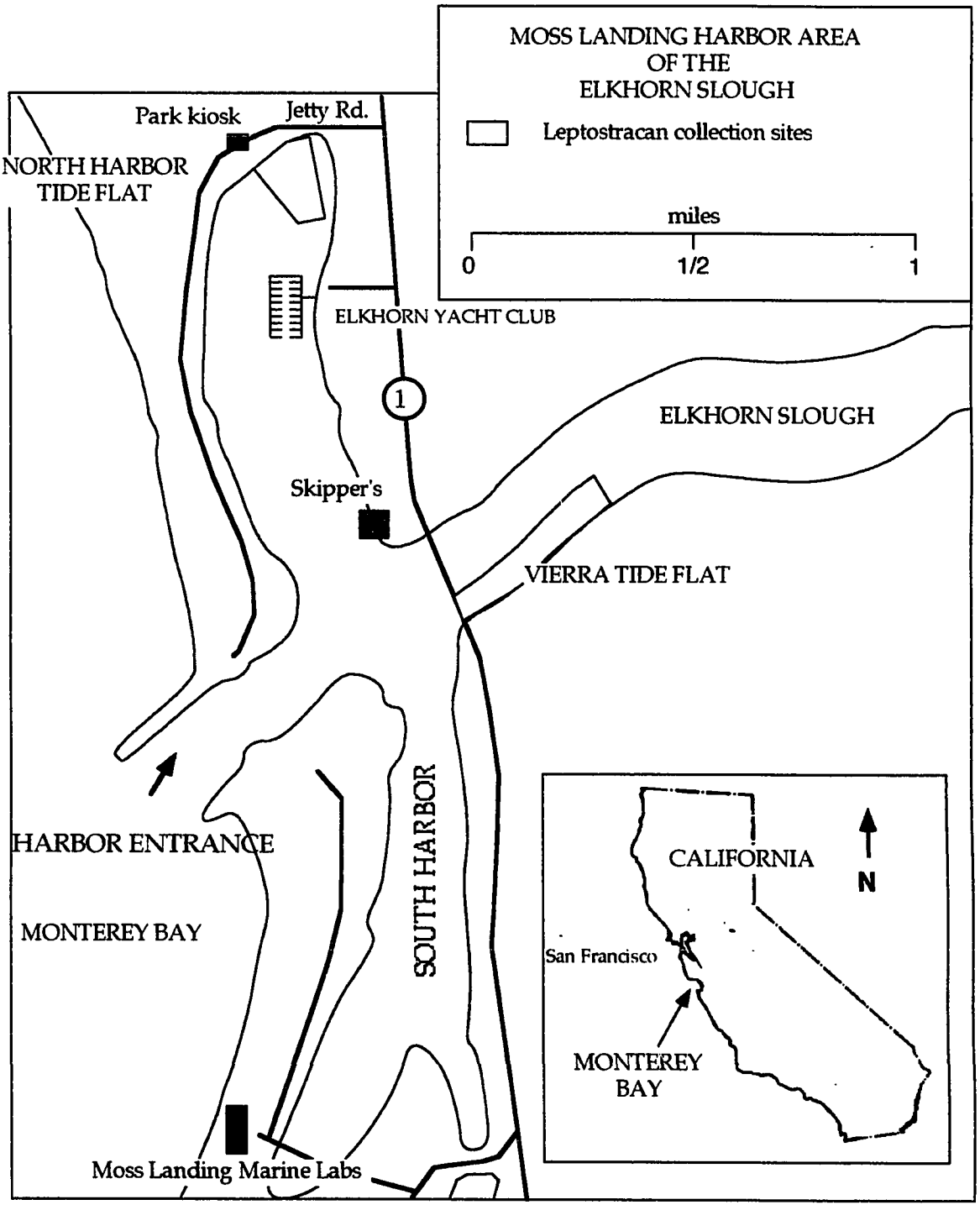

Figure 1. Map shows leptostracan collection sites on the North Harbor and Vierra tide flats located at the mouth of Elkhorn Slough in Moss Landing, CA.

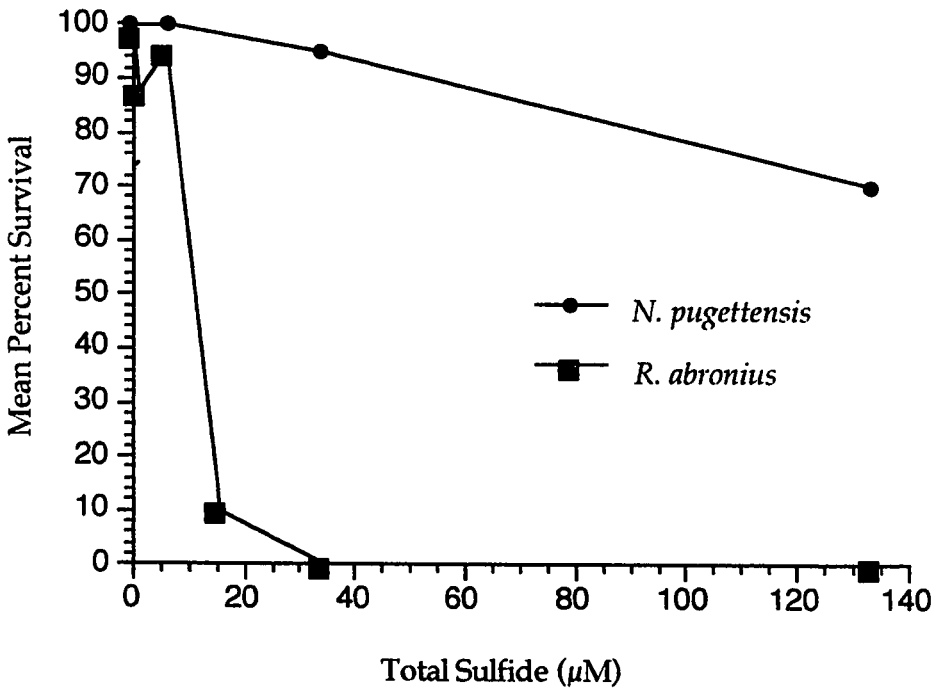

Figure 2. Response of *Nebalia pugettensis* and *Rhepoxynius abronius* to sulfide in the absence of sediment. (N=2)

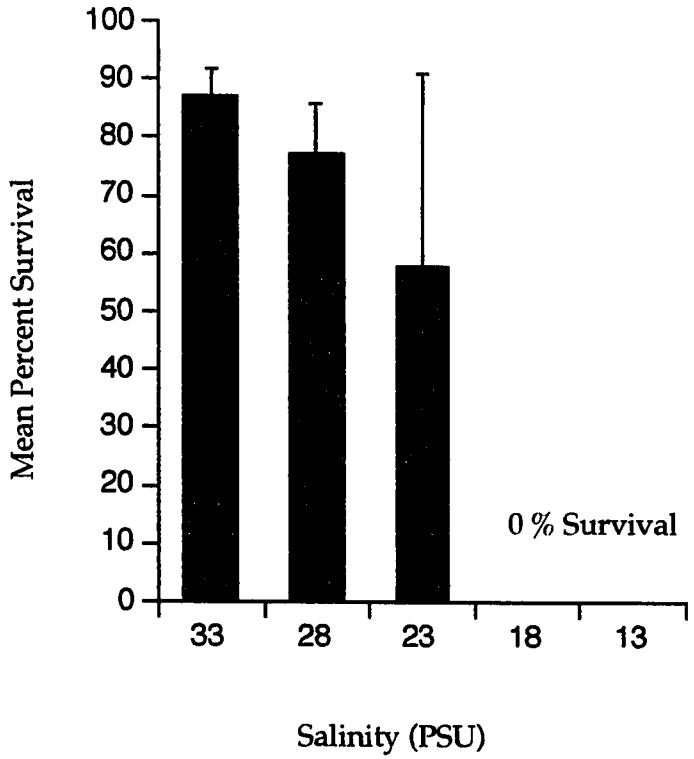

Figure 3. Mean percent survival of *N. pugettensis* in a ten day salinity tolerance test in which five salinities were tested. Error bars represent standard deviation. (N=3)

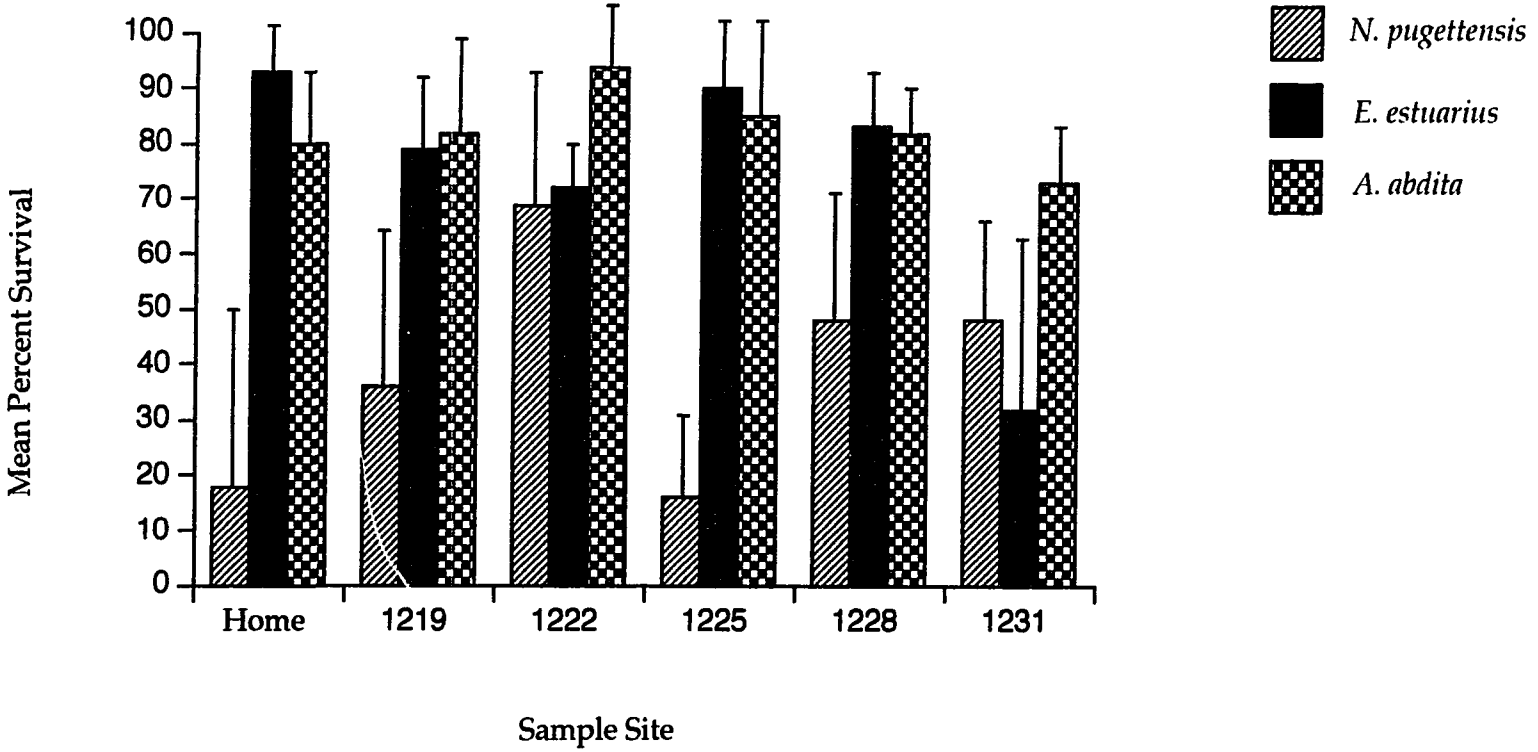

Figure 4. Mean percent survival of *Nebalia pugettensis, Eohaustorius estuarius* and *Ampelisca abdita* in a ten day reference sediment test with sediment samples collected from five sites in San Francisco Bay (Leg 31). Error bars represent standard deviation. (N=5) Leptostracans utilized in this test were cultured in laboratory aquaria for approximately 11 months prior to testing.

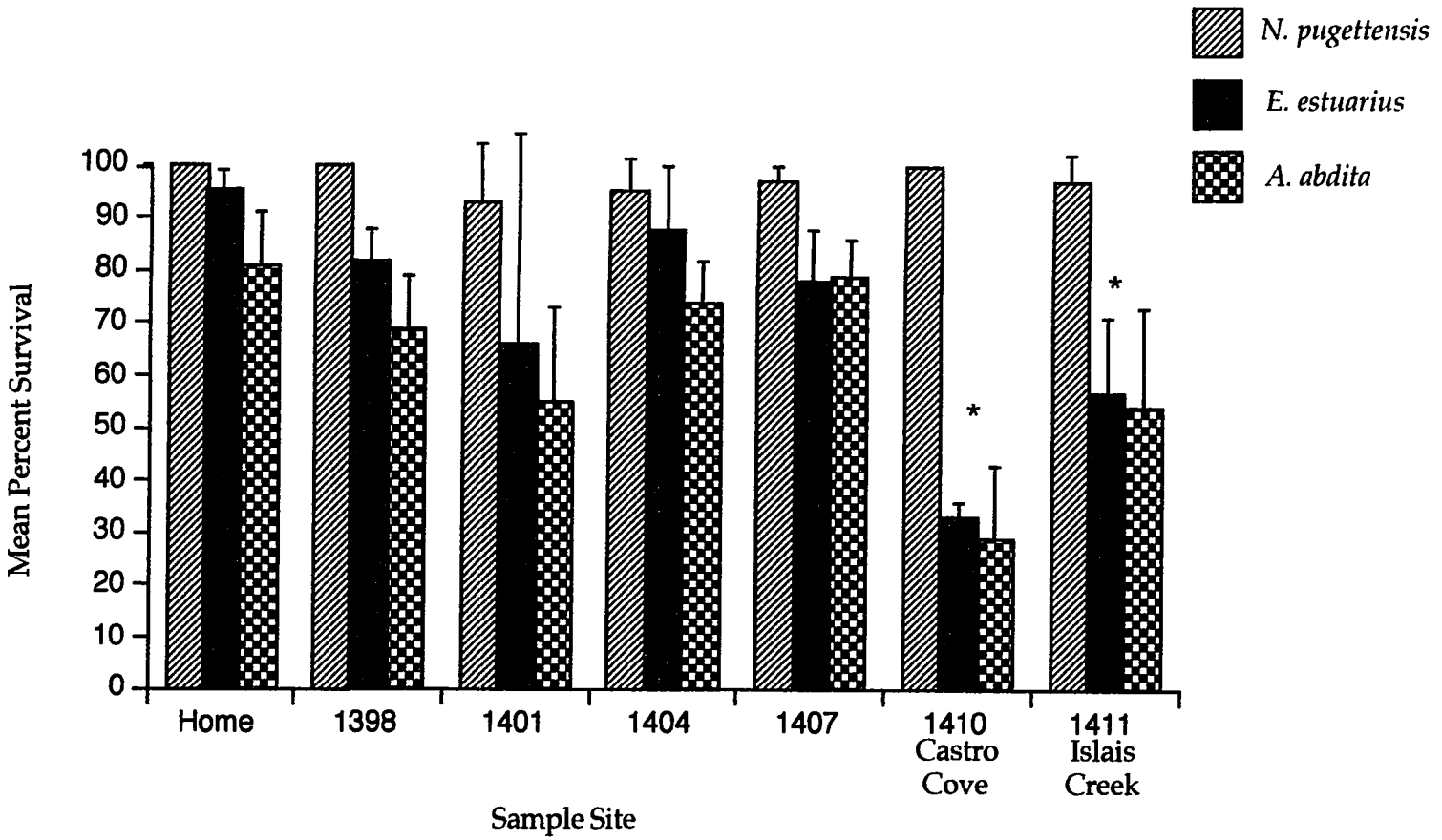

Figure 5. Mean percent survival of *Nebalia pugettensis, Eohaustorius estuarius* and *Ampelisca abdita* in a ten day reference sediment test with sediment samples collected from six sites in San Francisco Bay (Leg 35). Error bars represent standard deviation. (N=5) Leptostracans utilized in this test were cultured in laboratory aquaria for approximately two months prior to testing.

* Significant difference from home sediment. (p=.05)

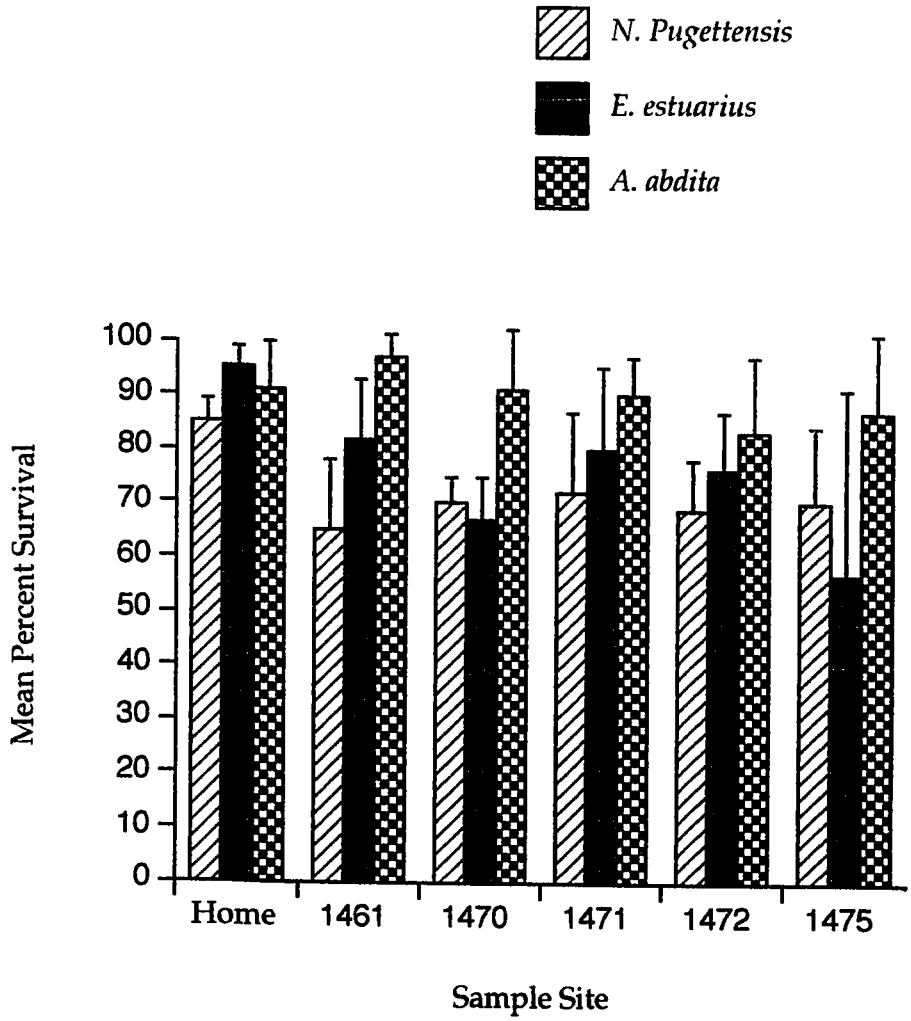

Figure 6. Mean percent survival of *Nebalia pugettensis, Eohaustorius estuarius* and *Ampelisca abdita* in a ten day reference sediment test with samples collected from five sites in San Francisco Bay (Leg 37a). Error bars represent standard deviation. (N=5) Leptostracans utilized in this test were cultured in laboratory aquaria for approximately six months prior to testing.

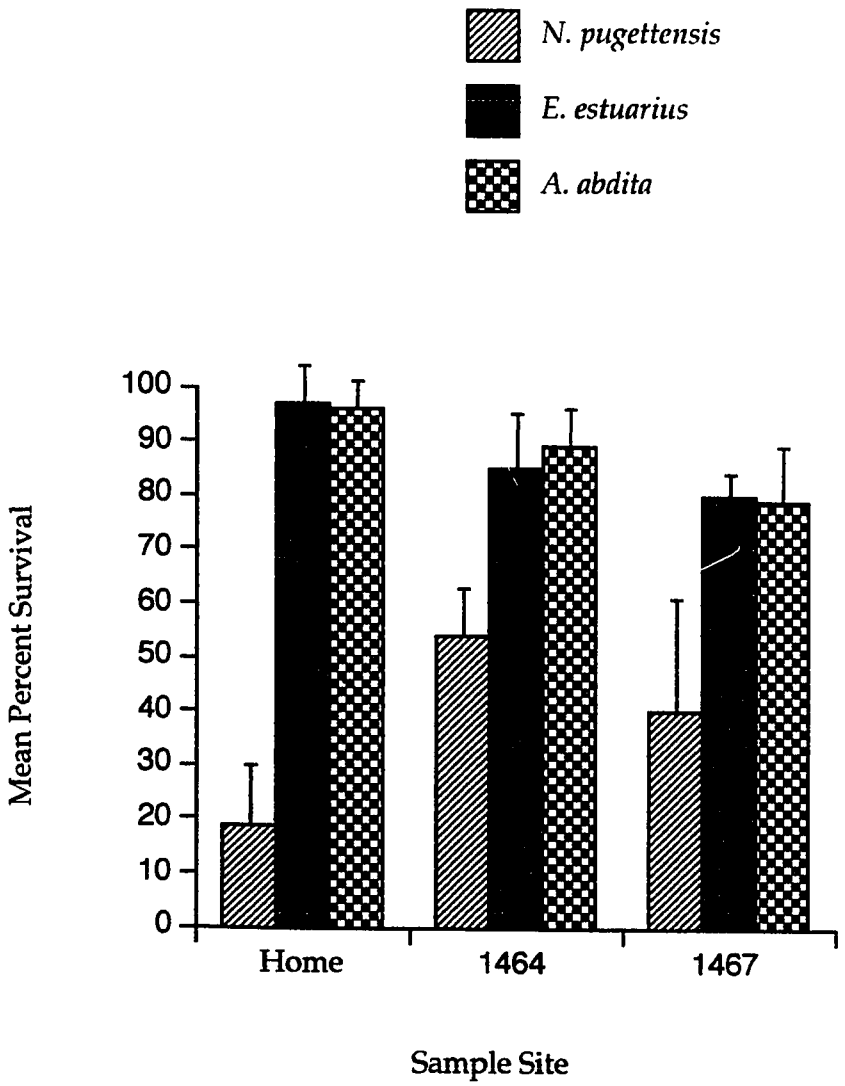

Figure 7. Mean percent survival of *Nebalia pugettensis, Eohaustorius estuarius* and *Ampelisca abdita* in a ten day reference sediment test with sediment samples collected from two sites in San Francisco Bay (Leg 37b). Error bars represent standard deviation. (N=5) Leptostracans utilized in this test were cultured in laboratory aquaria for approximately nine months prior to testing.

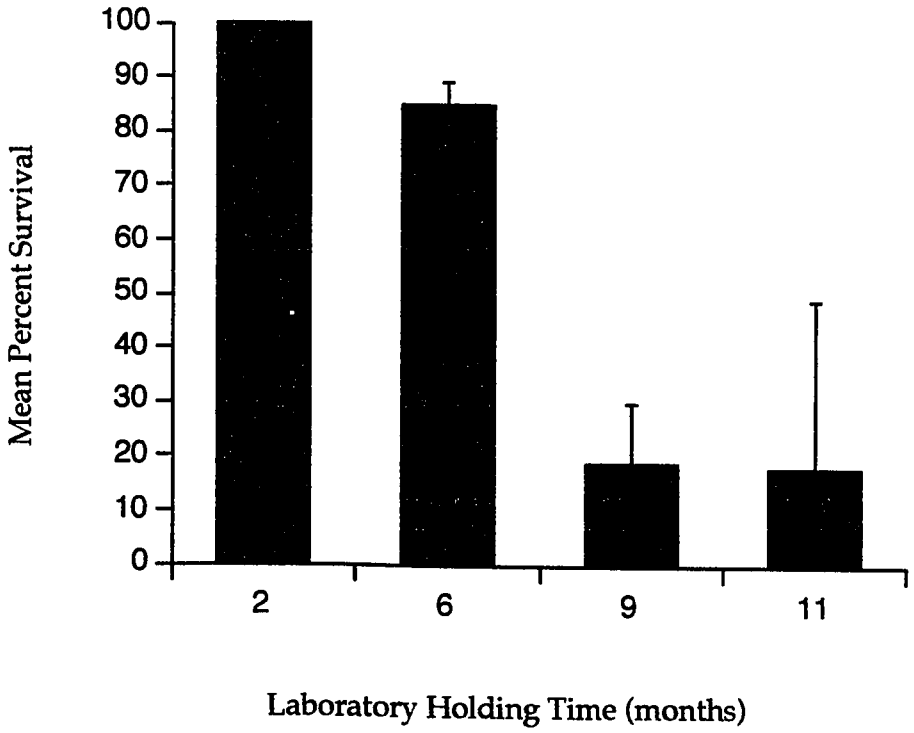

Figure 8. Mean percent survival of *Nebalia pugettensis* in four control treatments with leptostracans cultured in laboratory aquaria for two, six, nine and 11 months prior to testing. Error bars represent standard deviation. (N=5)

TABLE 1

Sieve and Hydrometer Analysis Results of the Fine Sediment Treatment from a Ten-day Grain Size Tolerance Test

Sediment Property	% Fraction	Total % Fines (% fines = % silt + % clay)
Coarse Sand	0.00	
Medium/Fine Sand	1.12	
Coarse Silt	13.18	98.88
Medium/Fine Silt	80.43	
Clay/Colloids	5.27	

TABLE 2
Cadmium LC_{50}'s from 96-Hour Water-Only Cd++ Reference Toxicant Tests with *Ampelisca abdita, Rhepoxynius abronius, Eohaustorius estuarius* and *Nebalia pugettensis*

Species	*Leg*	*Total Cadmium LC_{50} (mg/ liter)*	*95% Confidence Limits*
a) MPSL			
A. abdita	31	0.58	(0.44 - 0.77)
	35	0.67	(0.57 - 0.79)
	37a	1.00	(0.72 - 2.20)
	37b	0.45	(0.33 - 0.57)
R. abronius		0.80	(0.68 - 0.93)
E. estuarius	31	3.22	(1.28 - 8.11)
	35	8.10	(6.27 - 10.47)
	37a	9.65	(5.91 - 15.75)
	37b	4.84	(3.95 - 5.79)
N. pugettensis		1.32	(1.17 - 1.50)
	31	1.20	(1.07 - 1.36)
	35	1.72	(1.45 - 2.01)
	37a	1.58	(1.40 - 1.77)*
	37b	0.80	(0.64 - 1.00)*
		1.61	(1.38 - 1.87)*
b) ASTM			
A. abdita		0.33 (one test)	(0.29 - 0.38)
R. abronius		0.92	(0.68 - 1.25)
E. estuarius		9.33	(7.20 - 12.09)

a) MPSL = Data obtained from tests performed at the Marine Pollution Studies Laboratory during this study.

b) ASTM = Standard ranges obtained from the ASTM Standard Guide for Conducting Ten Day Sediment Toxicity Tests with Marine and Estuarine Amphipods (1990).

* Tests performed at 33 ppt. All other Cd^{++} tests were performed at 28 ppt.

TABLE 3

Survival of Various Test Species in Sediments from Five Presumably Toxic Sites in San Francisco Bay

Sediment Origin	Test Species	Mean % Survival	Significance (P= .05)
Castro Cove	*E. estuarius*	33% (2.7)	p= .0001 *
	A. abdita	29% (1.44)	p= .0002 *
	N. pugettensis	100%	NS **
Islais Creek	*E. estuarius*	57% (1.35)	p= .0002 *
	A. Abdita	54% (1.88)	p= .0106 *
	N. pugettensis	97% (4.5)	NS **
East China Basin	*E. estuarius*	5% (6.1)	p= .0001 *
	N. pugettensis	96% (4.2)	NS **
Guadalupe Slough	*N. pugettensis*	94% (5.5)	NS **
Lauritzen Channel	*N. pugettensis*	51% (17.8)	p= .0002 **

Mean percent survival and SD (in parentheses) are given. Statistical differences in survival in toxic sediments compared to control sediments were tested with t-tests. All proportion data were arcsine transformed to assure normal distribution. Results are presented in the far right column in the form of p-values. N = 5 for all samples; NS, not statistically significant.
* MPSL unpublished data
** This study

TABLE 4
Results of Feeding Protocol Tests One and Two with *N. pugettensis*

Treatment	*Mean Percent Survival*	*Standard Error*
a) Test One		
Control	48.3	± 6.2
One Feeding	36.7	± 9.4
Two Feedings	48.3	± 6.2
Four Feedings	41.7	± 8.5
b) Test Two		
Control One	98.3	± 2.4
Control Two*	96.6	± 4.7
One Feeding (4mg)	96.6	± 4.7
One Feeding (2mg)	96.6	± 4.7
Two Feedings (2mg)	98.3	± 2.4

a) Test One was performed with leptostracans cultured in laboratory aquaria for over 11 months.

b) Test Two was performed with leptostracans freshly collected from the field.

* Control Two contained 10 leptostracans per replicate, whereas all other treatments contained 20 per replicate.

APPENDIX A

Generation Time Experiments and Natural History Information

INTRODUCTION

Leptostracans (Crustacea; Malacostraca) have been studied and observed by scientists throughout the world (Table 5). Not only are leptostracans ubiquitous geographically, but they also occupy a diverse array of marine habitats. These habitats include shallow-water seagrass communities, sandy continental shelf areas and mud flats, and deeper regions such as Scripps Canyon and Rockall Trough. One recently described species, *Dahlella caldariensis* is common at hydrothermal vents on the Galapagos spreading center and the East Pacific Rise at 2,620 m (Hessler 1984).

Although confusion exists for species level taxonomy, six genera of the order Leptostraca are currently recognized (Dahl 1985). The genera include *Nebalia, Paranebalia, Sarsinebalia, Nebaliella,* the single holopelagic genus *Nebaliopsis* and the new genus associated with vent communities *Dahlella* (Dahl 1985 and Hessler 1984). The subject of this study, *Nebalia pugettensis*, is found on shallow intertidal mud flats from Puget Sound, Washington to Baja, California. The following Appendix discusses generation time data calculated in the laboratory for *N. pugettensis*. Natural history information obtained from observations accumulated during collection attempts for the previous study is also included.

Methods for Generation Time Calculation

Generation time of *N. pugettensis* was determined in two isolation experiments performed in the laboratory. All of the organisms utilized in these experiments were collected from mud flats located at the mouth of Elkhorn Slough in Moss Landing, California (Figure 1). On April 9, 1994, ten females with full brood pouches were isolated from a culture aquarium into one liter beakers. Each beaker contained one female, one liter ambient sea water (33 ppt) and one small piece of the green alga *Ulva sp.* Test beakers were placed in an ambient temperature sea water bath and temperature was recorded weekly. Weekly observations of egg size and color, juvenile development, and release of brood were recorded. After brood release, the parent female was removed. Juveniles from this generation were labeled generation one (G-1). Age of sexual maturity was recorded by noting the appearance of eggs in G-1 females and reproductive hooks on G-1 males. Males develop sexually dimorphic hooks on their second antennae when mature (Figure 9). In order to calculate a mean generation time, the ages of G-1 females were recorded at the release of their broods. To insure water quality, sea water was renewed weekly. Tetramin ® flake fish food was added to the beakers after each renewal.

This procedure was repeated on July 7, 1994 with an additional ten brooding females freshly collected from Elkhorn Slough. Each female contained peach colored eggs when isolated (Figure 11). The observations and calculations performed in experiment one were repeated. Three additional measurements were made: the number of G-1 juveniles released from each brood was counted and a mean calculated; the length (mm) of the original

female parent was measured within seven days of brood release and a mean calculated; and the lengths (mm) of five G-1 juveniles from each brood were measured within seven days of brood release and a mean calculated. Length was measured between the proximal ends of the rostral plate and the caudal furcae (taken from Rainer and Unsworth 1991).

Generation Time Results

In the first generation time experiment, a total of seven G-1 females developed eggs: two from beaker three, two from beaker six and three from beaker seven. Juveniles from all other beakers died before development of eggs or hooks. The mean age of the seven G-1 females at the first appearance of eggs was 111.43 ± 21.2 days (Table 6). The mean generation time (age of G-1 female at release of brood) was 135 ± 17.9 days (Table 6). A mean brood retaining time was calculated as 19.23 ± 2 days by the following equation:

Generation Time - First Appearance of Eggs = Brood Retaining Time

Mean age at the first appearance of reproductive grasping hooks on the second antennae of males was 68.3 ± 7.3 days (Table 6). Again, only males from beakers three, six and seven developed hooks.

In the second generation time experiment, a total of ten G-1 females developed eggs; one from beaker four, two from beaker five, two from beaker six and five from beaker ten. In all other beakers no eggs were observed. The mean age of the ten G-1 females at the first appearance of eggs in this experiment was slightly higher at 134.4 ± 14.8 days (Table 6). Mean generation time was also higher in the second experiment at 159 ± 13.6 days (Table 6). A

mean brood retaining time of 24 ± 3.9 days was calculated (Table 6). Mean age at the first appearance of reproductive grasping hooks on the second antennae of males was lower in experiment two at 42.9 ± 1.7 days (Table 6). In experiment two, hooks were observed in all ten beakers, whereas hooks were only observed in the three beakers in which eggs were also observed in experiment one.

Three additional observations were recorded in the second generation experiment: mean number of juveniles released from each brood, the length of juveniles within seven days of brood release, and the length of the parent female within seven days of brood release. The mean number of juveniles released from the brood pouch of each of the original ten brooding females was 53.6 ± 11.5 (Table 6). Juveniles averaged 1.39 ± .1 mm in length within seven days of release, with the parent female averaging 6.7 ± .3 mm (Table 6).

DISCUSSION

Habitat Description

The ability of members of the order Leptostraca to survive low oxygen tensions is well known (Thiele 1926/27). *Nebalia pugettensis* is most commonly found in oxygen-poor, organically rich sediments (Clark 1932, Brusca and Brusca 1990). On mud flats located at the mouth of Elkhorn Slough, Moss Landing, CA, *N. pugettensis* was also commonly found in fine, organically rich sediments. Dense aggregations of *N. pugettensis* were observed under thick mats of *Ulva* in the black, sulfide-rich, anoxic mud. They were commonly found in the top few centimeters of sediment beneath the *Ulva*. When associated with algal mats, *Nebalia sp.* have been reported to

exist in higher densities than *Nebalia sp.* existing in neighboring bare sand habitats (Vetter 1995). Vetter (1995), reported densities of more than three million leptostracans and amphipods per square meter of decaying kelp and surfgrass mats in a submarine canyon off La Jolla, California. The animals in these mats eat the detritus and, in turn, provide food for many species of fish.

In this study, dense populations of *N. pugettensis* were associated with algal cover. Leptostracans were collected at various times over a two year period beginning July 19, 1993. Seasonal changes in algal cover were observed during this period. These changes coincided with leptostracan availability and ease of collection. Algal cover was highest from June to August, at which time, large numbers of *N. pugettensis* were observed and collected. Algal cover decreased as a result of higher water motion caused by winter storms, a seasonal decline in nutrients, and changes in irradiation. Coverage completely disappeared at times during January, February and March. *N. pugettensis* collection attempts during periods of low to absent algal cover resulted in few to zero leptostracans observed.

Since *N. pugettensis* is a detritus feeder, a thick algal layer could provide an abundant source of detritus for food and presumably support denser populations of leptostracans. *N. pugettensis* leaves the sediment when mating (personal observation). The *Ulva* layer may therefore, not only provide food, but act as a protective covering against predation and water motion during mating. The visible decrease in population of *N. pugettensis* in the absence of *Ulva* may be due to this loss of a food source and protective covering.

Reproductive Cycle

N. pugettensis broods its young from egg to full development as a juvenile within the carapace (see plates 9-13). No planktonic or pelagic stages were observed in the reproductive cycle of *N. pugettensis* in this study. Juveniles were well developed, averaging 1.4 mm in length when released from the brood pouch(Table 6). Not only are juveniles well developed at release (Plate 13), but an individual brooding female releases an average of 54 juveniles with each brood (Table 6). A single female has been observed to produce at least three broods in the laboratory before dying (Eric Vetter, personal comm.). Mating and reproduction appear to occur throughout the year in laboratory cultures. It is likely that brooding females are readily available at all times in the field as well. Generation times of 135 and 159 days were calculated in generation time experiments one and two, respectively (Table 6). Approximately 4-5 months after being released from the brood pouch, an individual female is capable of releasing its first brood. Consecutive broods are probably formed faster.

Ecological Strategies

Throughout this study, *N. pugettensis* was the dominant organism observed under dense *Ulva* patches on the Vierra and North Harbor mud flats. Because of the relatively high *N. pugettensis* population densities, these patches were selected as collection sites. In the fringing areas of these patches, Tanaids and Gammarid amphipods appeared to replace leptostracans as the dominant organisms. During a leptostracan collection in early March 1995, the Tanaid *Leptochelia dubia* was the predominant organism observed on the

North Harbor mud flat. Leptostracans had been extremely abundant in November in these same areas, but none were found in the March collection attempt. Again, algal cover and density were visually lower in March than in November and sediments appeared "cleaner" with less algal cover.

It appears that the dense populations of *N. pugettensis* observed in this study were confined to organically rich, low-oxygen sediments formed beneath thick *Ulva* patches. However, some species of leptostraca are commonly found in clean sandy habitats, although in densities 100 times lower than *Nebalia sp.* found in neighboring algal mats (Vetter 1995). It may be that Tanaids and Gammarid amphipods outcompete leptostracans in the cleaner, more oxygenated microhabitats of the mud flats observed in Elkhorn Slough. Because *N. pugettensis* is significantly larger than these other organisms and more visible because of its opaque color, it may be preferentially preyed upon by intertidal fishes, especially in areas with less protective algal cover.

Summary

Nebalia pugettensis populations exhibited variation in both temporal and spatial abundance. The temporal abundance pattern, consisting of high densities in the summer and low densities in the winter, was associated with algal cover. The thick *Ulva* cover typical in summer, probably acts as food source and protective covering for leptostracans emerging from the sediment. This food supply and protective cover appears to be a requirement for the development of populations observed in the summer.

The spatial abundance pattern, forming obvious patches of intensely high populations, was associated with patches of dense *Ulva* cover and the associated organically rich, oxygen poor sediments beneath. Leptostracans appeared to dominate these anaerobic habitats, while amphipods appeared to dominate more aerobic habitats. Tolerances for low oxygen and elevated sulfide levels allow *N. pugettensis* to utilize this niche.

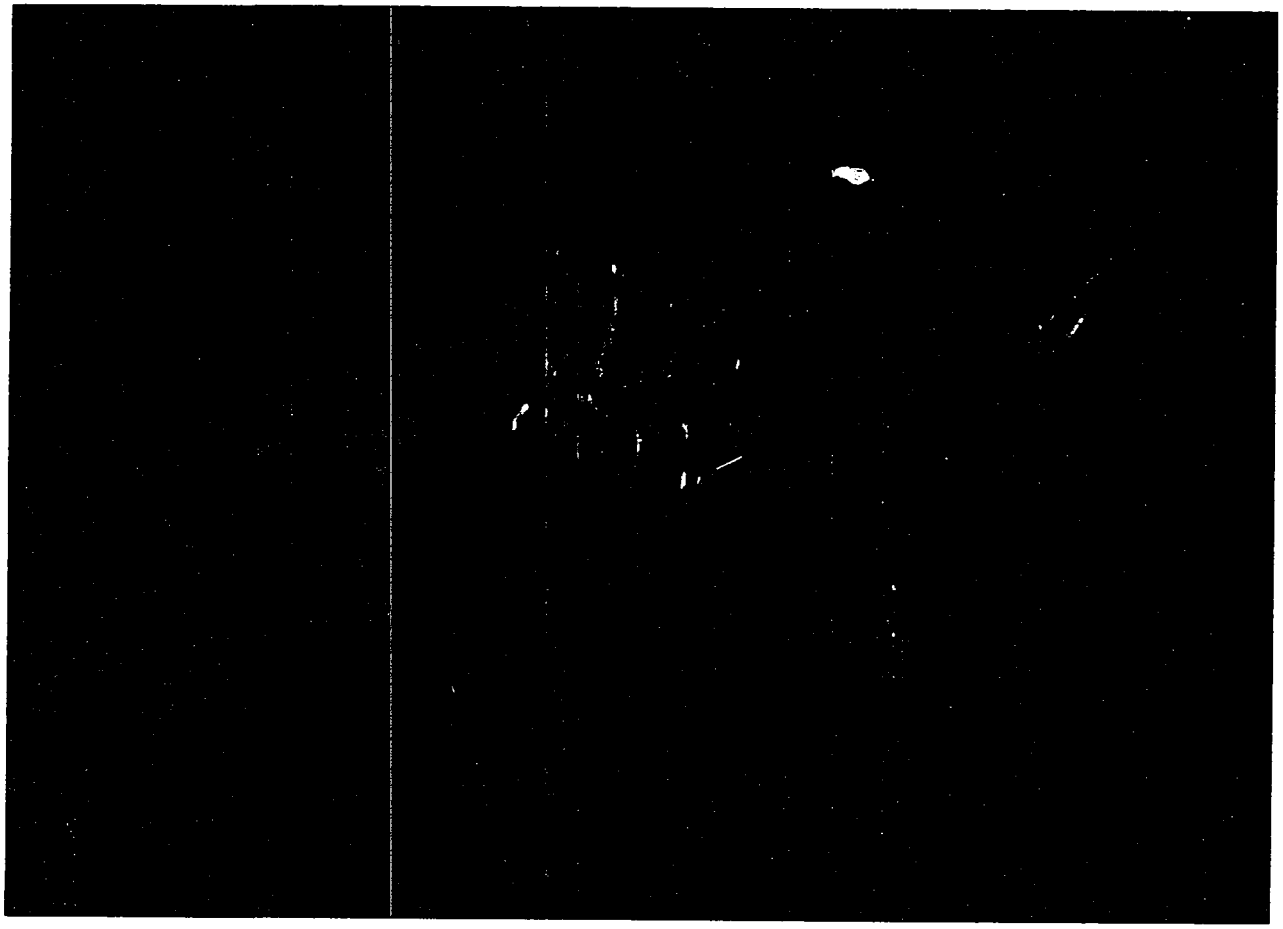

Figure 9. Male leptostracan, *N. pugettensis*, with sexually dimorphic curved hooks located on the second antennae.

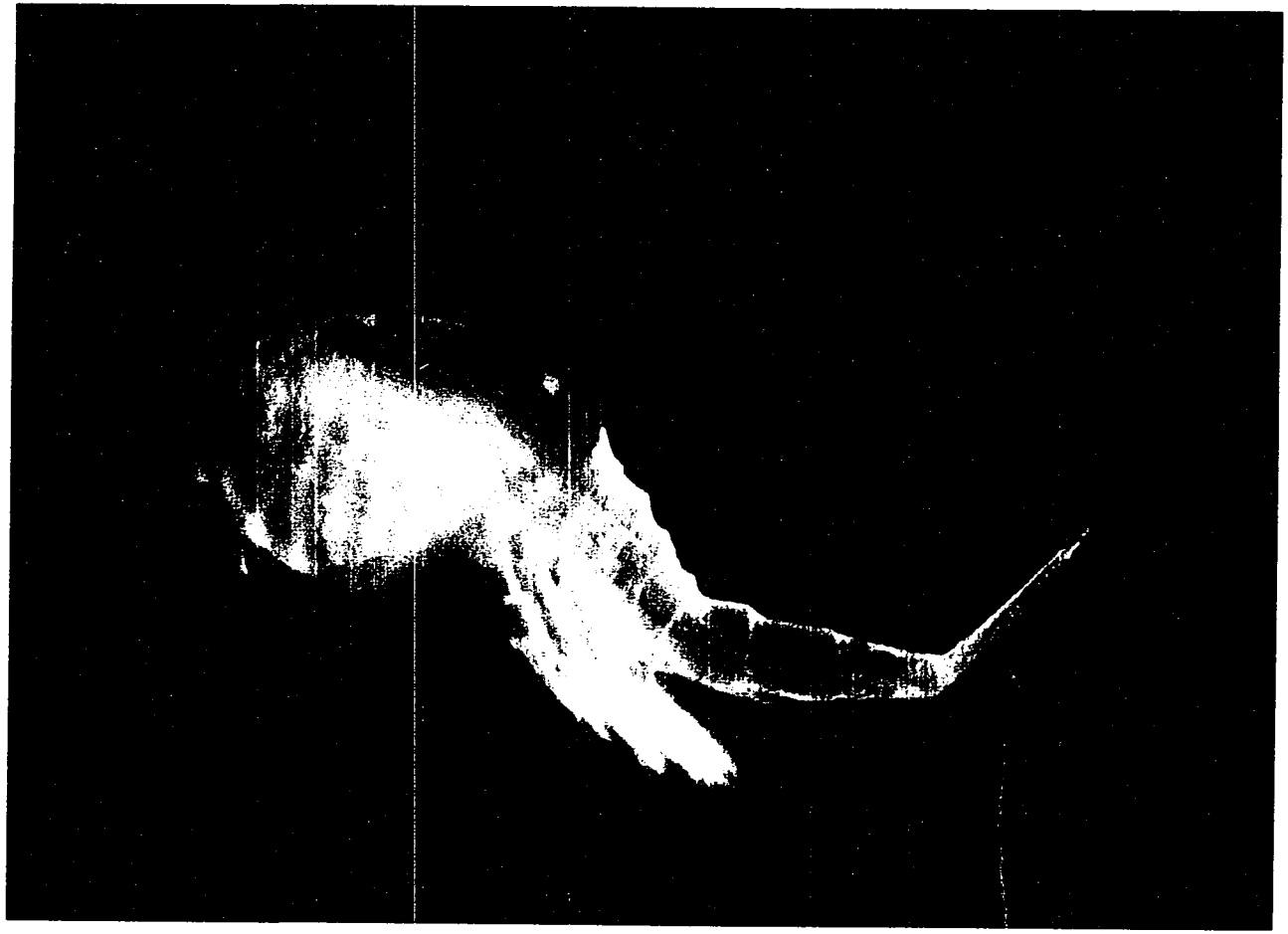

Figure 10. Female leptostracan, *N. pugettensis*,with light colored round distinct eggs, approximately five days into the developmental cycle.

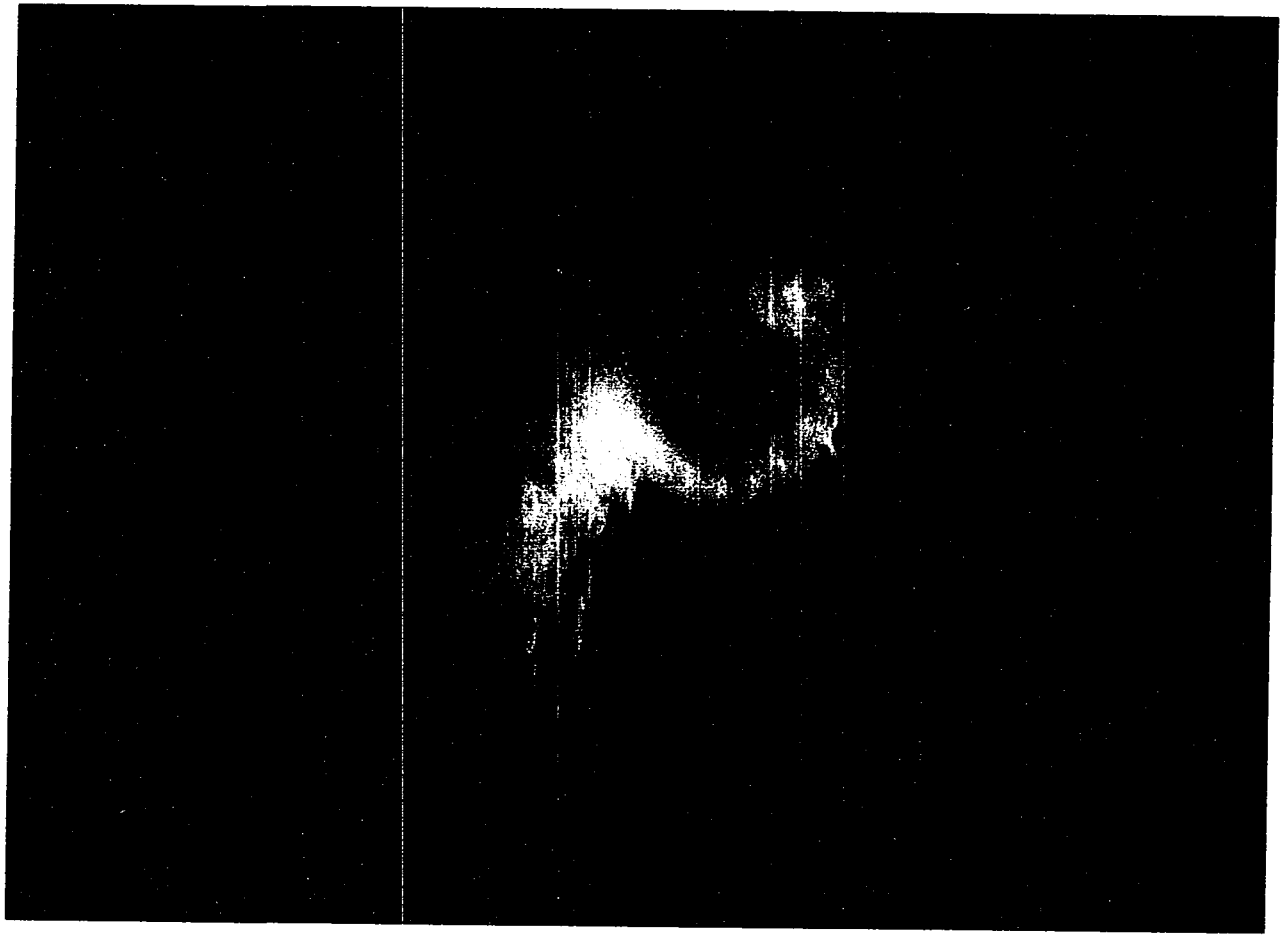

Figure 11. Female leptostracan, *N. pugettensis*, with dark colored round distinct eggs, approximately ten days into the developmental cycle.

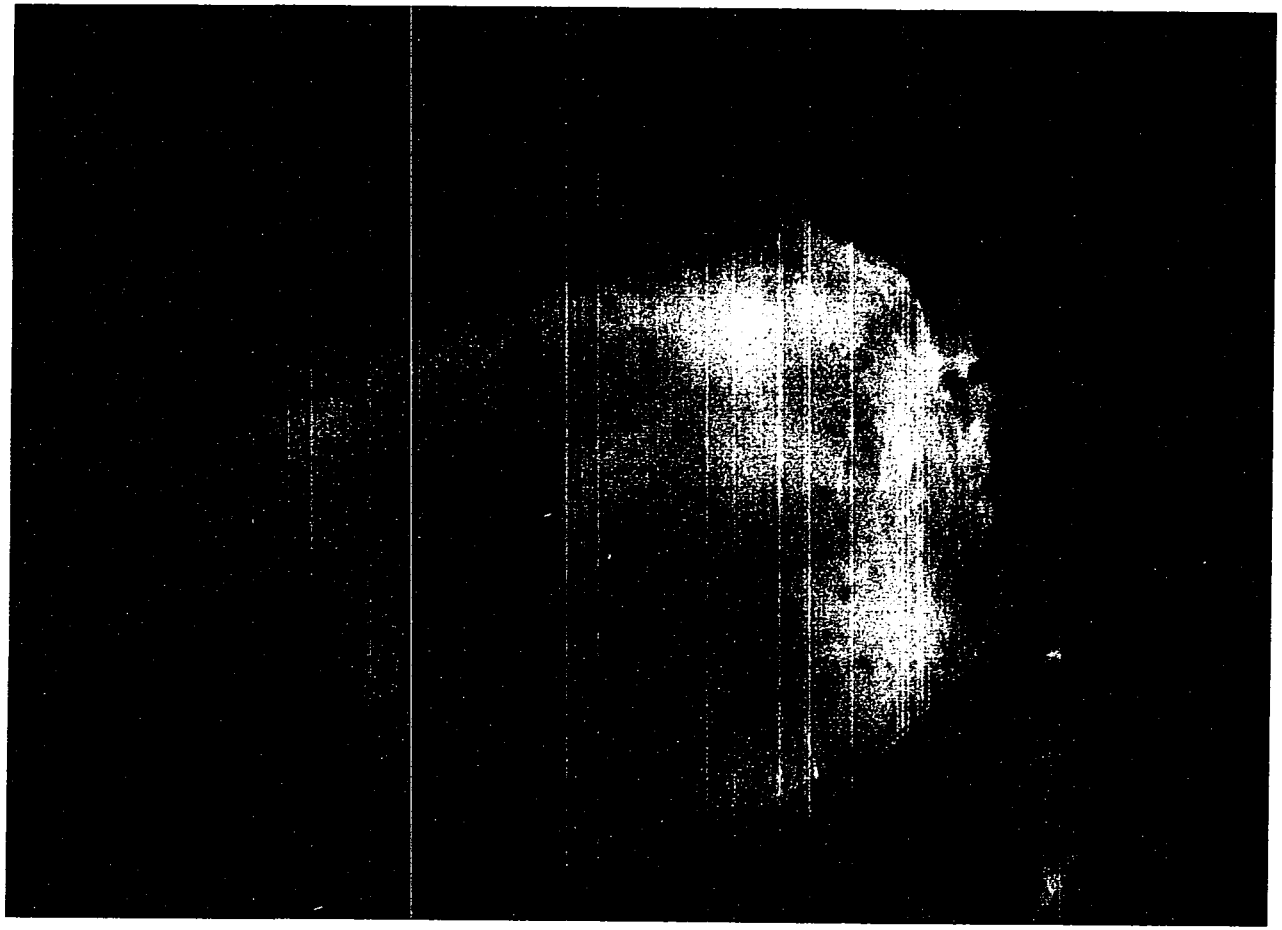

Figure 12. Close-up of female leptostracan, *N. pugettensis*,brood pouch. Carapace contains developing juveniles, approximately 20 days into the developmental cycle. Eyespots of the juveniles are visible through the carapace.

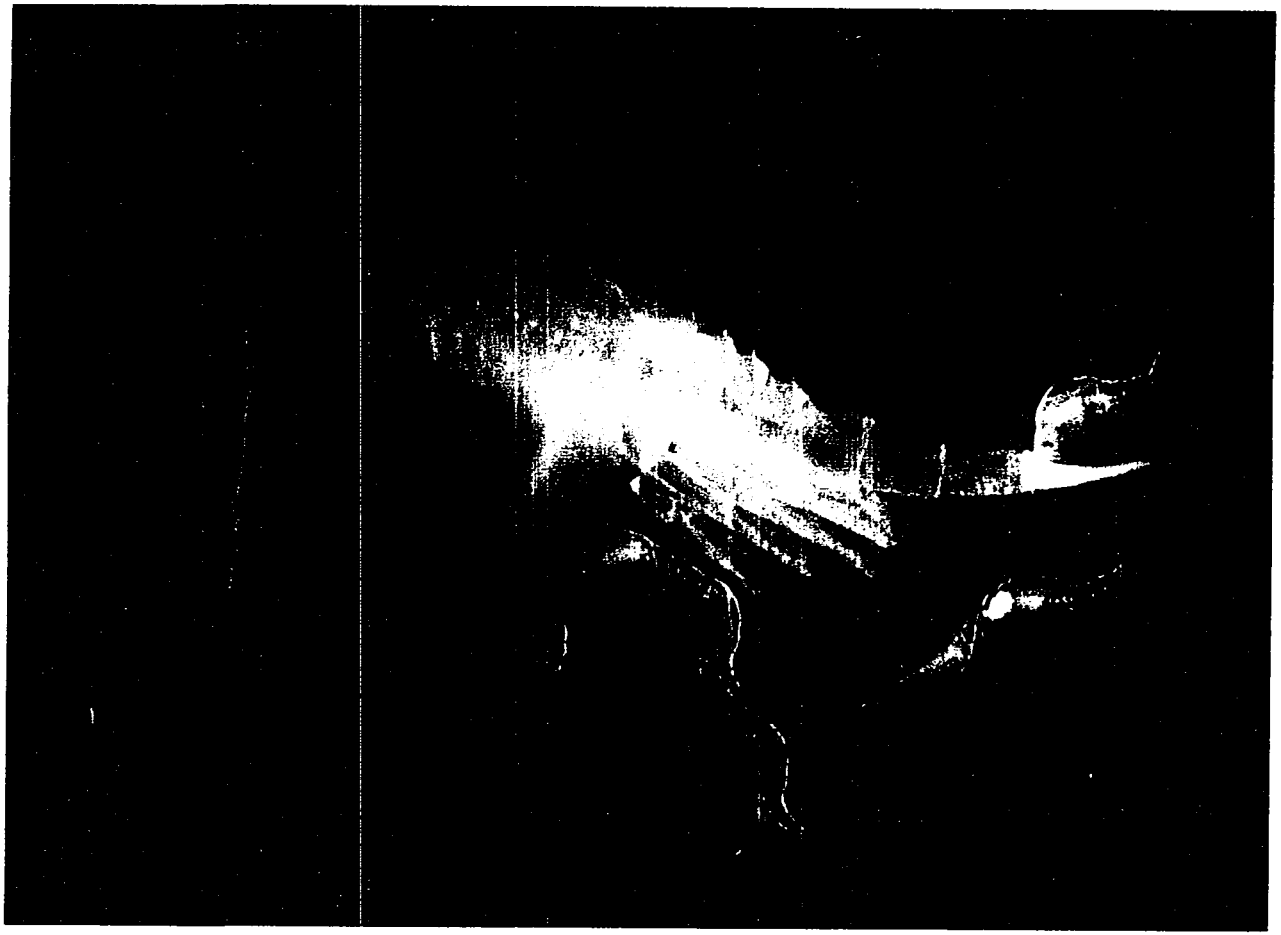

Figure 13. Female leptostracan, *N. pugettensis*, with newly released juveniles (within three minutes).

TABLE 5

Summary of References Describing the Geographic Range of the Order Leptostraca

Geographic Location	*Reference*
Brazil	Wakabara 1965
Australia	Rainer and Unsworth 1991
Magellan region Antarctic region Falkland Islands	Dahl 1990
Northeastern United States	Hessler and Sanders 1965
Pakistani Coast of the Northern Arabian Sea	Kazmi and Tirmizi 1989
Rockall Trough	Mauchline and Gage 1983
British Columbia	Conlan and Ellis 1979
Bahamas and Southern Florida	Brattegard 1970
European Continental Shelf	Dahl 1985
Scripps Canyon, Southern California	Eric Vetter personal comm.
Elkhorn Slough, Northern California	This Study

TABLE 6
Summary of Generation Time Experiments One and Two

Description	*Experiment One (age in days)*	*Experiment Two (age in days)*
a) First Appearance of Eggs	111.43 ± 21.2	134.4 ± 14.8
b) Brood Retaining Time	19.23 ± 2	24 ± 3.9
c) Generation Time	135 ± 17.9	159 ± 13.6
d) First Appearance of Hooks	68.3 ± 7.3	42.9 ± 1.7
e) Size of Juvenile at Release (mm)	no data	1.39 ± .1

a) First Appearance of Eggs = age of female when the first set of eggs appeared.
b) Brood Retaining Time = length of time from egg, to full development and release of the juveniles from the brood pouch.
c) Generation Time = First Appearance of Eggs + Brood Retaining Time
d) First Appearance of Hooks = age at the first appearance of sexually dimorphic hooks located on the second antennae of males.
e) Size of Juvenile at Release = length of juveniles (mm) when released from the brood pouch (within three days). Measurements were taken between the proximal ends of the rostal plate and the caudal furcae.