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Survey of Biological Effects of Toxicants Upon Puget Sound Biota. II. Tests of Reproductive Impairment

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(Continued on last page)

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P.M. Chapman, D.R. Munday, J. Morgan,
and R. Fink
E.V.S. Consultants Ltd.
North Vancouver, B.C., Canada

R.M. Kocan and M.L. Landolt
Fish and Wildlife Health Consultants
Suquamish, Wash.

R.N. Dexter
E.V.S. Consultants Inc.
Seattle, Wash.

Rockville, Md.
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U.S. DEPARTMENT OF COMMERCE
Malcolm Baldrige, Secretary

National Oceanic and Atmospheric Administration
John V. Byrne, Administrator

National Ocean Service
R.Adm. Kelly E. Taggart, Acting Assistant Administrator

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DISCLAIMER

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

A variety of chemical contaminants have been found in Puget Sound. These chemicals often occur in complex mixtures and in varying concentrations from place to place. The biological implications of these mixtures was largely unknown, though various biological disorders appeared to co-occur with the highest concentrations of certain chemical groups. In 1982 the MESA (Marine EcoSystems Analysis) Puget Sound Project supported a survey of the toxic effects of samples taken from 97 sites in the Sound. The results of direct-exposure bioassays showed geographic trends in toxicity; the most toxic samples were from the highly industrialized parts of the Sound, the least toxic samples were from remote areas. The study reported here is a direct follow-on to the initial survey. The purpose was to verify the results of the initial survey and to determine if the reproductive success of marine biota could be impaired by direct exposure to samples of Puget Sound sediments. The Contract Officer's Technical Representative from the MESA Puget Sound Project Office for this study was Edward R. Long.

EXECUTIVE SUMMARY

Puget Sound has been the subject of intensive chemical, oceanographic and biological study by the Marine Ecosystems Analysis (MESA) Puget Sound Project. Assessment of the health of Puget Sound has previously included chemical analyses of contaminant levels at different sites, and a broad-scale toxicity survey at 97 stations using a progression of bioassay tests (Chapman et al., 1982). The results of those studies indicated that a number of sites in Puget Sound had high contaminant levels and that sediments from those sites were capable of causing biological effects. The present study was initiated to determine if the conclusions from the broad-scale toxicity survey were supported by a broader range of biological tests on a smaller number of selected stations.

A total of 22 stations were chosen for study from the 97 used in the previous broad-scale toxicity survey. Composite sediment grab samples were collected and tested with four basic tests of reproductive impairment effects using a wide range of taxa and proven state-of-the-art techniques.

Study results were used to prioritize specific stations. On the basis of both the present study results and previous broad-scale toxicity surveys at these stations, the most toxic areas were Commencement Bay Waterways and the lower Duwamish River. Stations in Elliott Bay were of variable toxicity, with those off Alki Point and Denny Way showing greatest toxicity and those off other waterfront areas showing least. Samples from Sinclair Inlet were of high toxicity while outer Commencement Bay stations showed low toxicity. The reference site (Port Madison) was among the least toxic but did exhibit some effects.

Oyster larvae bioassays were conducted with Crassostrea gigas by exposing fertilized eggs to settled sediment slurries for 48 h then determining the number of live larvae and any abnormalities. A total of 18 stations demonstrated significant abnormalities or mortalities.

Cell reproduction studies were conducted by exposing rainbow trout gonad (RTG-2) and bluegill fry (BF-2) cells to sediment extracts during logarithmic growth. The RTG-2 cells have an active mixed function oxidase (MFO) enzyme system capable of metabolizing otherwise inert xenobiotics, whereas the BF-2 cells do not. All stations induced significantly reduced cell growth in RTG-2 cells, and 5 stations also induced reduced growth in BF-2 cells.

The development of surf smelt (Hypomesus pretiosus pretiosus) was monitored from fertilized eggs to larvae during exposure to settled sediment slurries. Deleterious effects observed included reduction in hatching success, reduction in larval survival, and premature hatching. A total of 20 stations demonstrated some adverse effect on development.

Life-cycle studies were conducted with the polychaete worm Capitella capitata by raising the worms from newly hatched trochophore larvae to adults in sediments and in sediment elutriates. Deleterious effects were observed including mortality, inhibition of growth and egg laying, and abnormal larval metamorphosis. A total of 15 stations demonstrated some adverse effect on the worms' life-cycle.

The results of this study substantiate the findings of a previous broad-scale toxicity survey which included these stations (Chapman et al., 1982), and provide additional information for classifying the level of biological activity at each station. There were only 3 stations where completely different results were recorded between the two studies. One station previously described as non-toxic was shown to be toxic while 2 stations were shown to be less toxic than previously described. The differences noted at these three stations may reflect the use of different tests and organisms and/or the patchiness of sediment toxicity. The broad-scale survey was undertaken with single grab samples from each station while the present study involved collection and testing of composite samples which provided a more representative measure of the selected stations. Comparisons between the two studies indicate that the approaches used have successfully described a variety of biological responses that serve to rank contaminant-related effects among the stations.

ACKNOWLEDGEMENTS

The study team would like to express their appreciation for the assistance and encouragement provided by Mr. E. Long, the Contracting Officer's Technical Representative. We also thank Mr. D. Misitano of the National Marine Fisheries Service and Mr. J. Cummins of EPA Region X for the provision of technical advice.

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1.0 INTRODUCTION

The intent of the MESA Puget Sound Project is to develop an understanding of the existing concentrations, fate and effects of environmental contaminants in Puget Sound. Although a number of studies have shown high environmental levels of chemicals in different areas of the Sound, and it has been shown that a variety of biological disorders (e.g. tissue damage to fish and shellfish, changes in benthic community structure) occur in areas with high levels of contaminants (Long, 1982), it is only recently that direct evidence of toxicity from Puget Sound sediments has been provided.

A previous study (Chapman et al., 1982) was funded by NOAA with the objective of determining if Puget Sound sites induce specific, testable biological effects. This study involved a screening survey at 97 sites and was successful in demonstrating a number of toxic responses (respiration, lethal and cytogenetic effects). Following this study, 22 of the 97 original stations were resampled for further biological effects testing, with emphasis on tests of reproductive impairment. The present report documents the results of this additional testing which had the specific objective of determining if the conclusions from the screening tests (Chapman et al., 1982) could be supported by a broader range of biological tests.

The approach taken in the present study involved compositing sediment grab samples from each test site, and testing these samples for possible reproductive impairment effects using a wide range of taxa and techniques. The rationale for testing reproductive impairment effects was to examine possible implications to populations. Tests were concerned with: oyster larvae toxicity , surf smelt egg and larvae toxicity, polychaete life cycles, and in vitro cell reproduction. Results were used to further classify the comparative toxicity of sediment samples from various areas of Puget Sound.

1.1 Objectives

The specific objectives of this study were:

a. To reoccupy a total of 22 stations from Puget Sound, and obtain a composite sample consisting of aliquots from 5 Van Veen grabs from each site.

b. To determine the effects, if any, of exposure to these sediment samples on the reproductive processes of Puget Sound biota.

c. To determine whether the results of previous screening tests could be verified with a second series of tests, using impairment of reproductive success as the test criteria.

2.0 METHODS

2.1 Geographical Study Area

This study was intended to supplement previous broad-scale toxicity testing by performing additional and corroborative bioassays on sediments from 22 of the 97 stations sampled by Chapman et al. (1982). These 22 stations (Figs. 1-7) were selected primarily based on their previous toxic response. Numbering of stations corresponds to Chapman et al. (1982). Major sampling emphasis was in the three urbanized embayments of Elliott Bay and the Duwamish River, Commencement Bay and associated waterways, and Sinclair Inlet. A single station from Port Madison was incorporated as a non-urban reference site.

Precise station location information (e.g. latitude, longitude, water depth, position relative to shore facilities, etc.) is presented in Appendix A.

2.2 Approach

Acute lethality testing conducted by Chapman et al. (1982) showed that death was not a common response for adult animals exposed to contaminated sediments for 10 days. However, sublethal effects as indicated by changes in oligochaete respiration, and genotoxicity as indicated by mitotic abnormalities in cultured fish cells, did occur at many stations. The choice of stations for retesting included a majority of stations (13) where both genotoxicity and sublethal effects were noted, 8 stations where only one type of effect was noted, and one station (Station 42) which, despite being located in a chemically contaminated area, showed no significant toxicity (Table 1).

In the previous broad-scale survey (Chapman et al., 1982), single grab samples were taken at each of the 97 stations. In the present study, 5 replicate grab samples were collected at each station and composited to obtain a sample that was more representative of each station.

2.3 Relative Sensitivities of Tests and Organisms

Various researchers (as summarized by Birge et al., 1979) have shown that the developmental stages (eggs, larvae) of many fish and invertebrate species are particularly sensitive to trace contaminants (e.g. PCB's, chlorinated hydrocarbons, pesticides, metals). The reproductive potential of many species may be adversely affected by concentrations of toxic substances that are harmless or sublethal to most adult organisms.

To test whether this general observation was true in Puget Sound, a number of tests of reproductive impairment were initiated using sediment samples from previously-identified areas of biological effects. Four different tests were undertaken involving the use of representative Puget Sound biota of commercial or ecological importance: Pacific oyster larvae; surf smelt eggs and larvae;

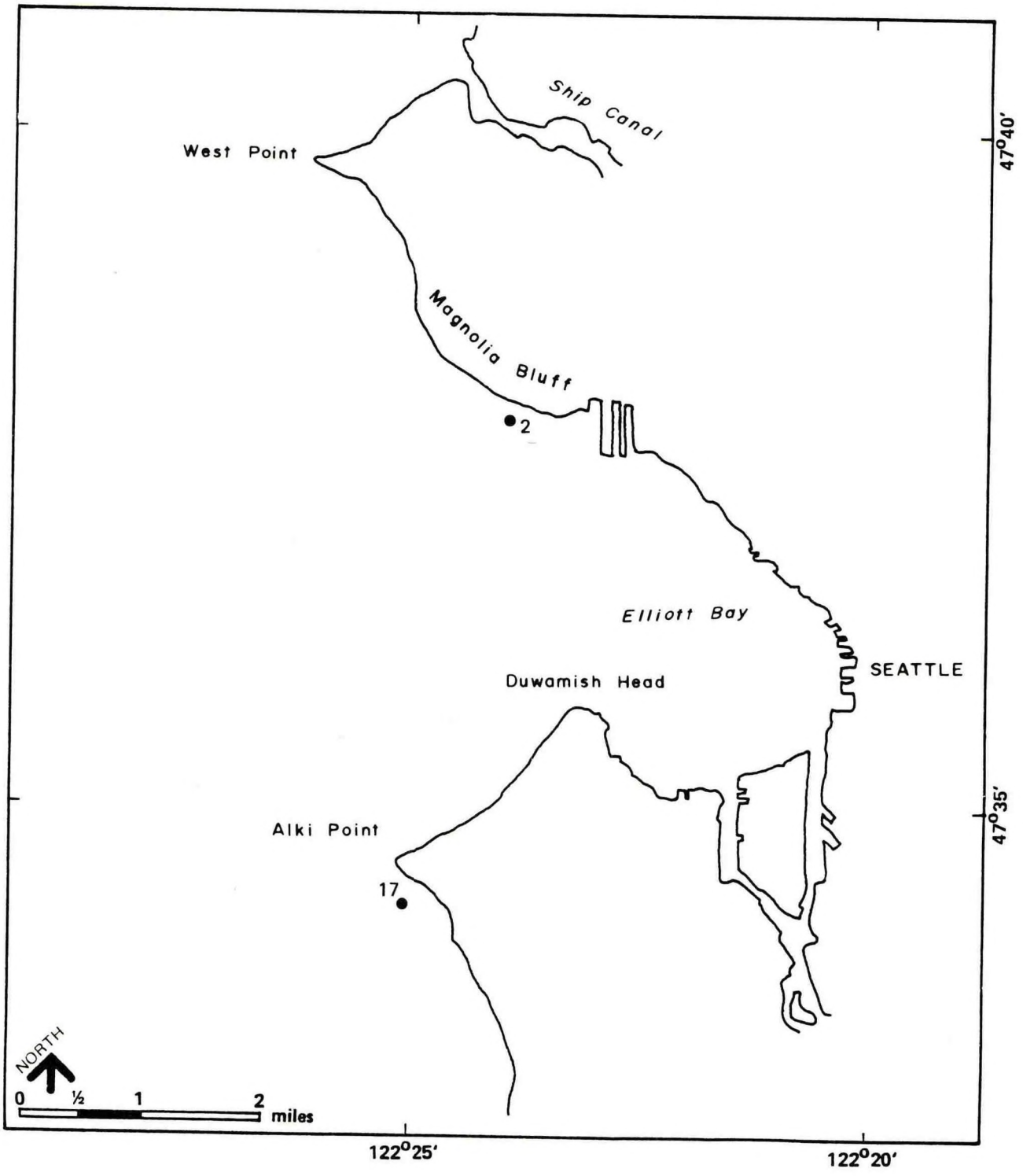


Figure 1. Station Locations at Alki Point and Magnolia Bluff.

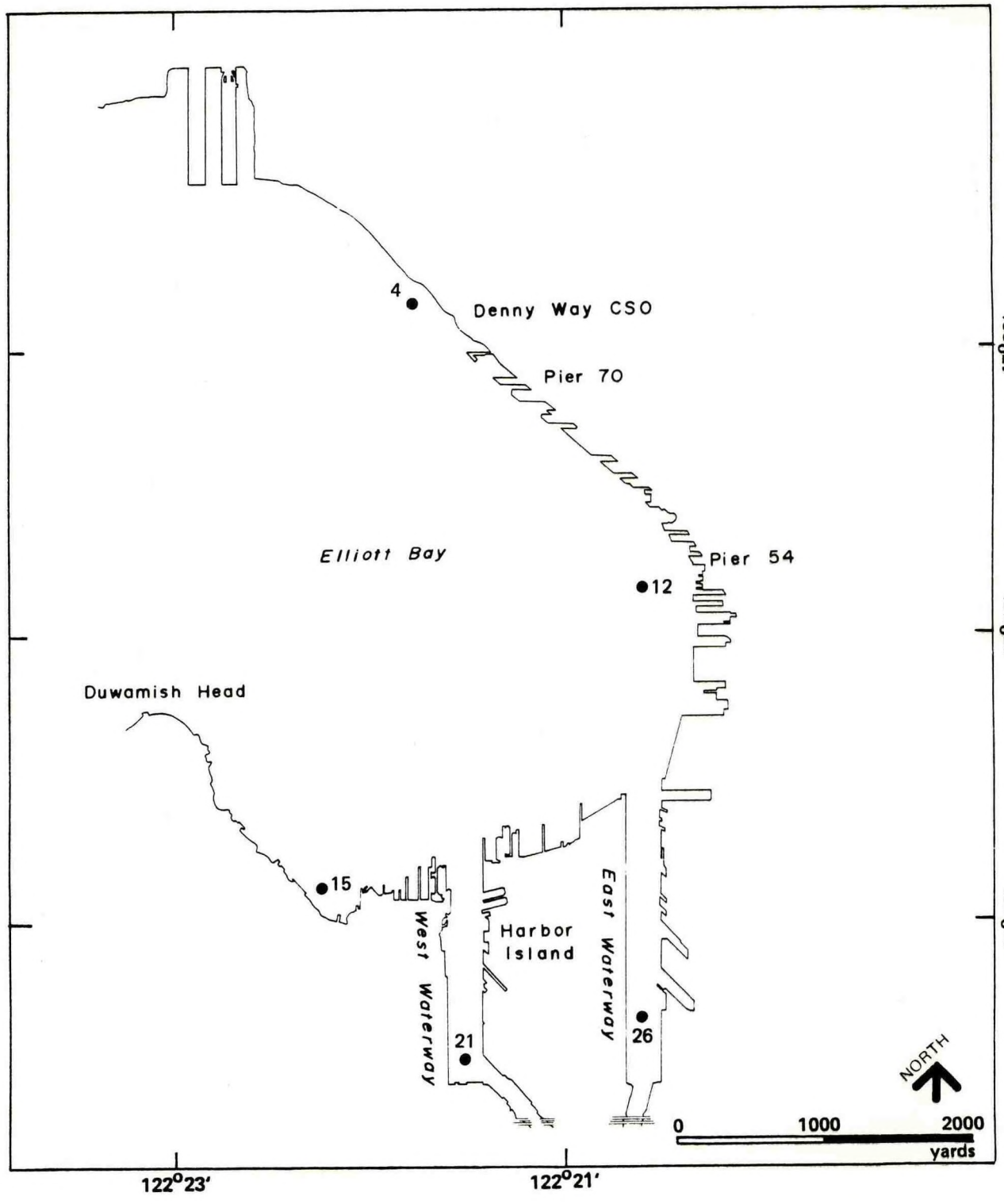


Figure 2. Station Locations in Elliott Bay and the Lower Duwamish River.

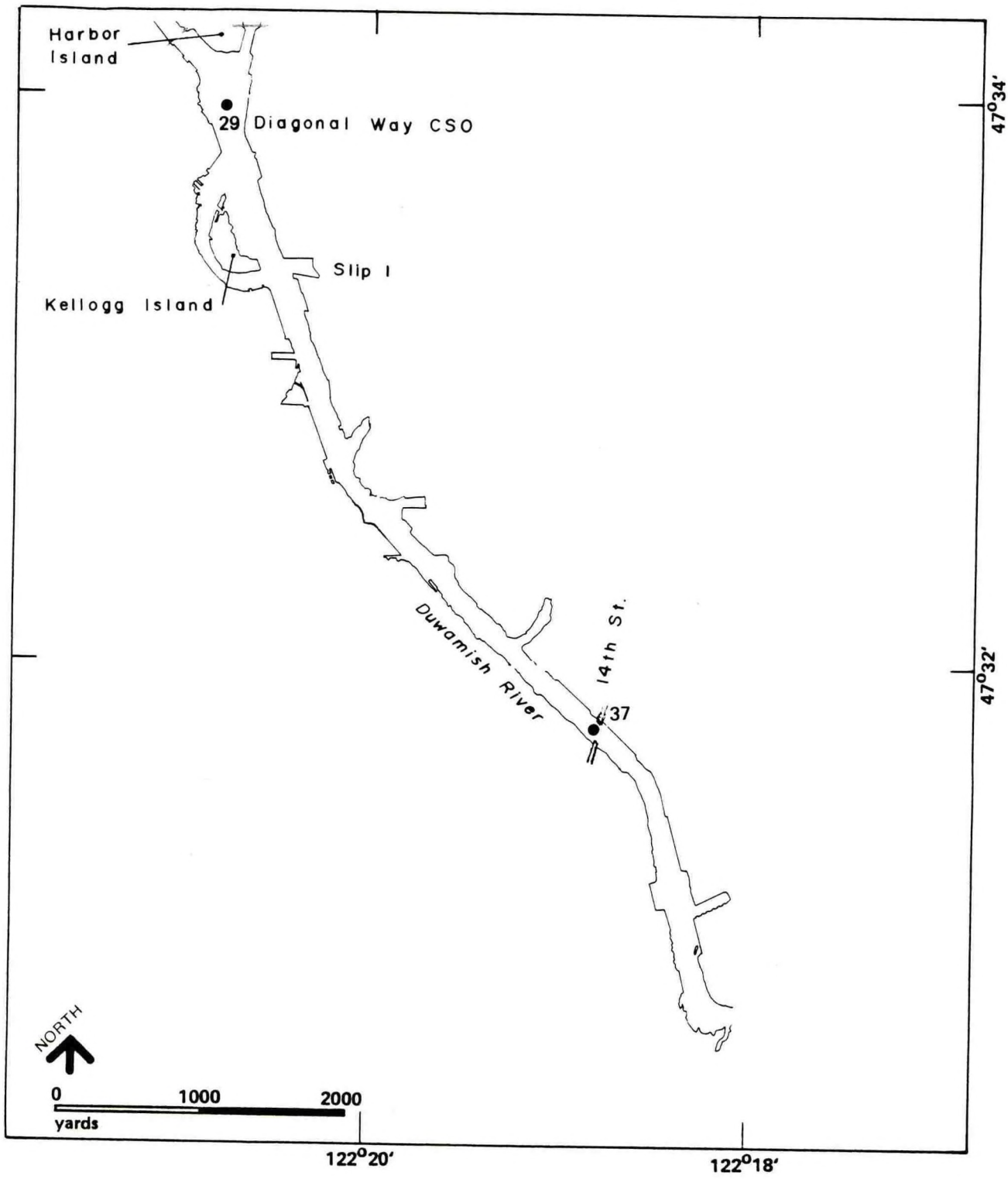


Figure 3. Station Locations in the Upper Duwamish River.

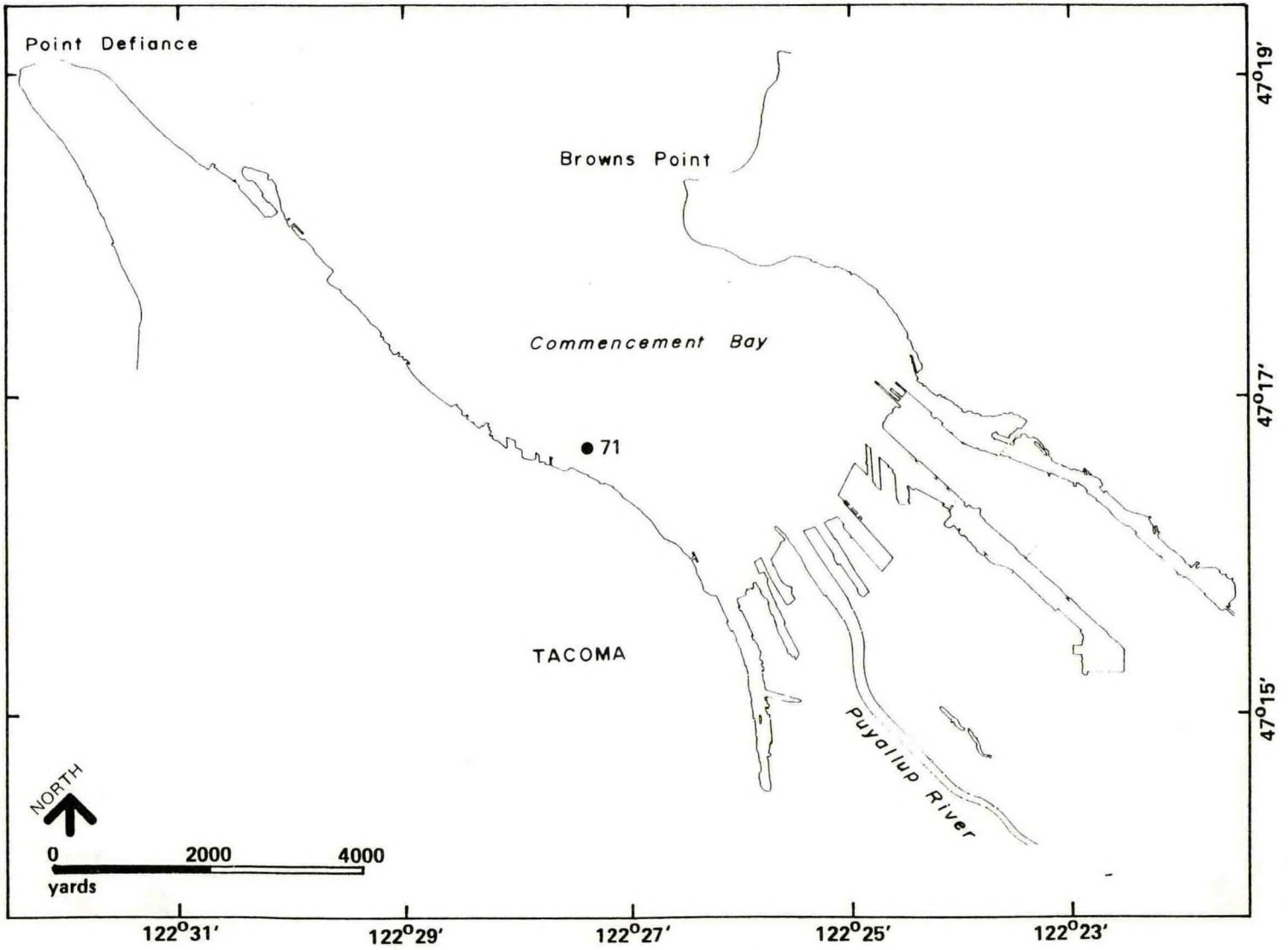


Figure 4. Station Location in Commencement Bay.

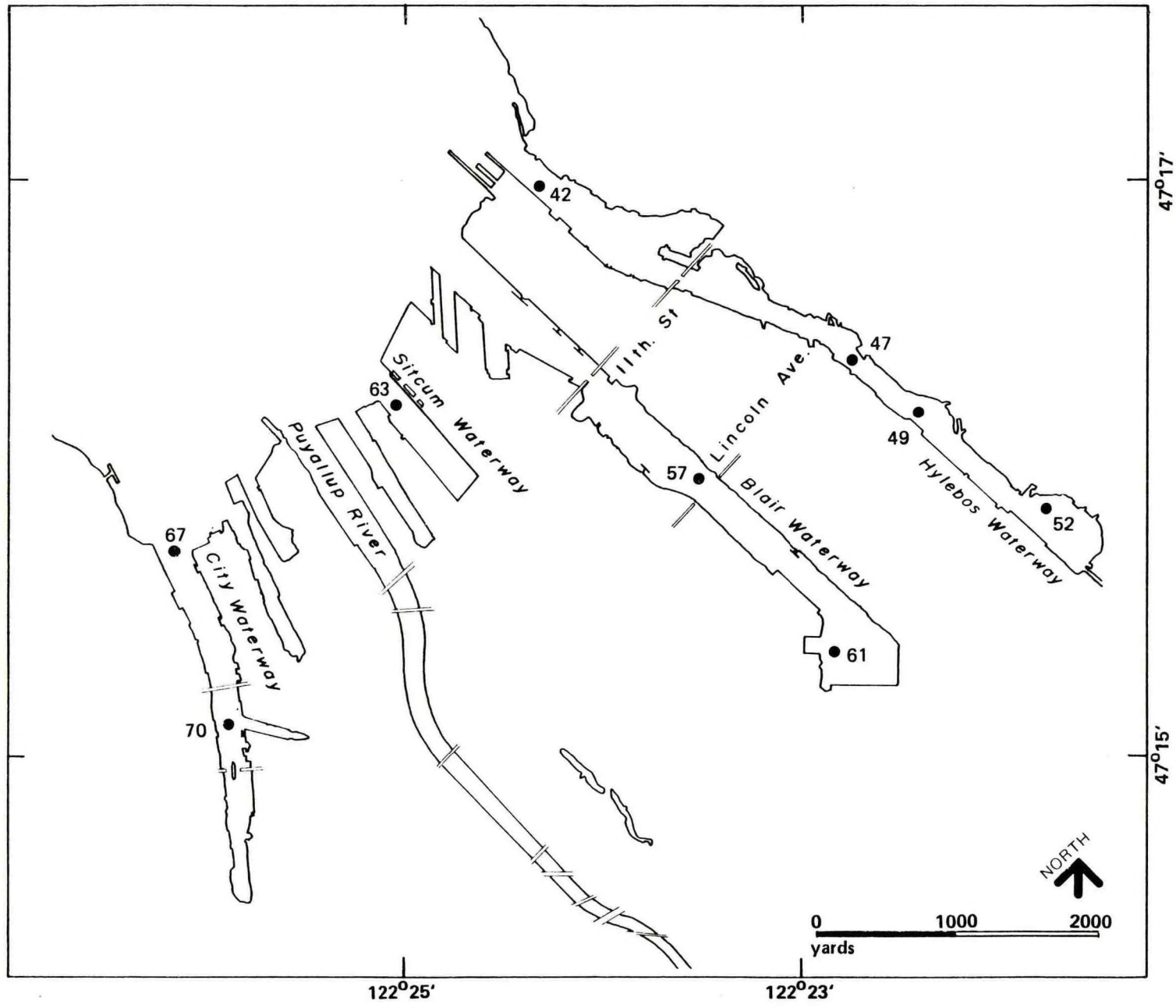


Figure 5. Station Locations in the Waterways of Commencement Bay.

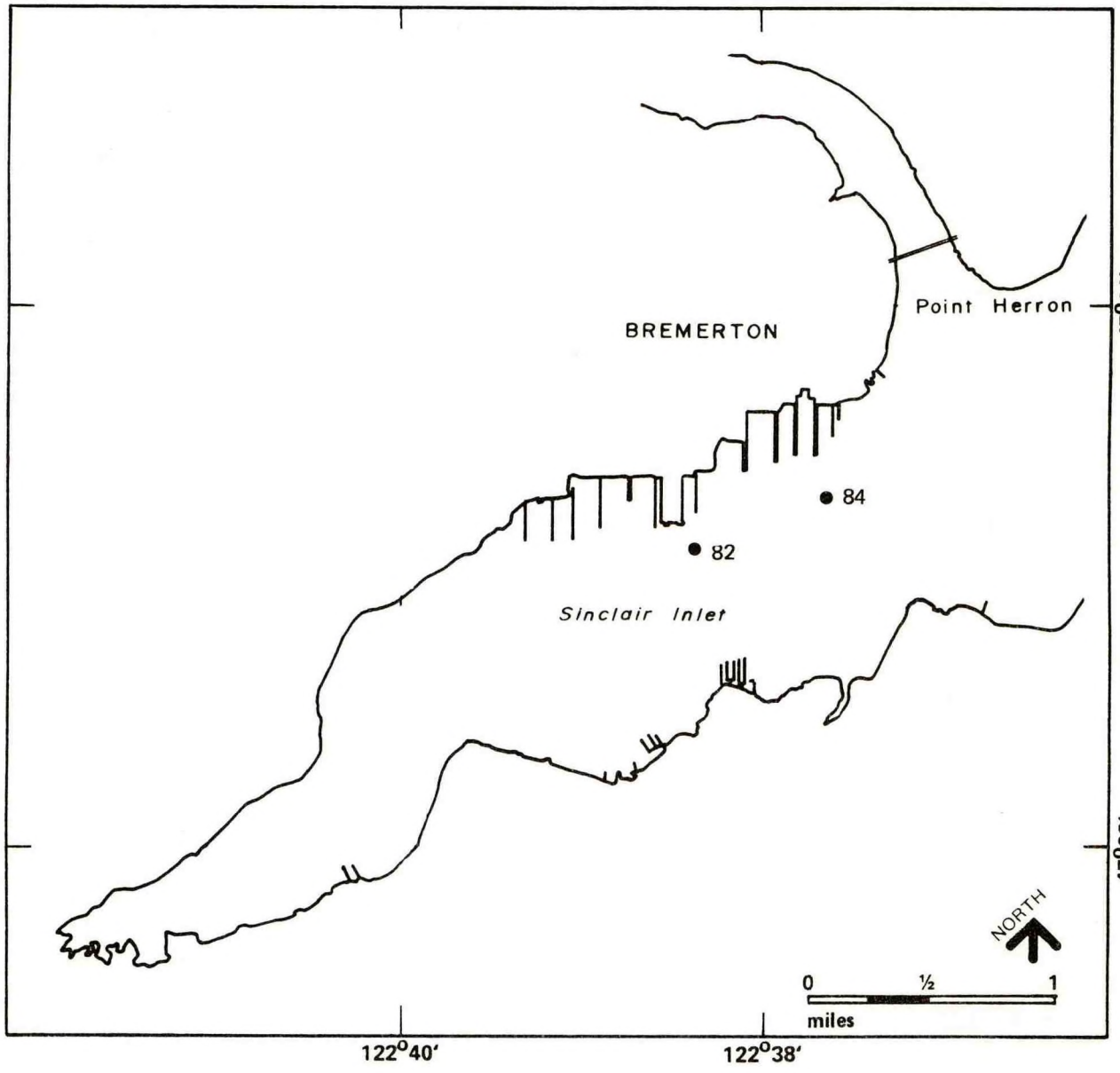


Figure 6. Station Locations in Sinclair Inlet.

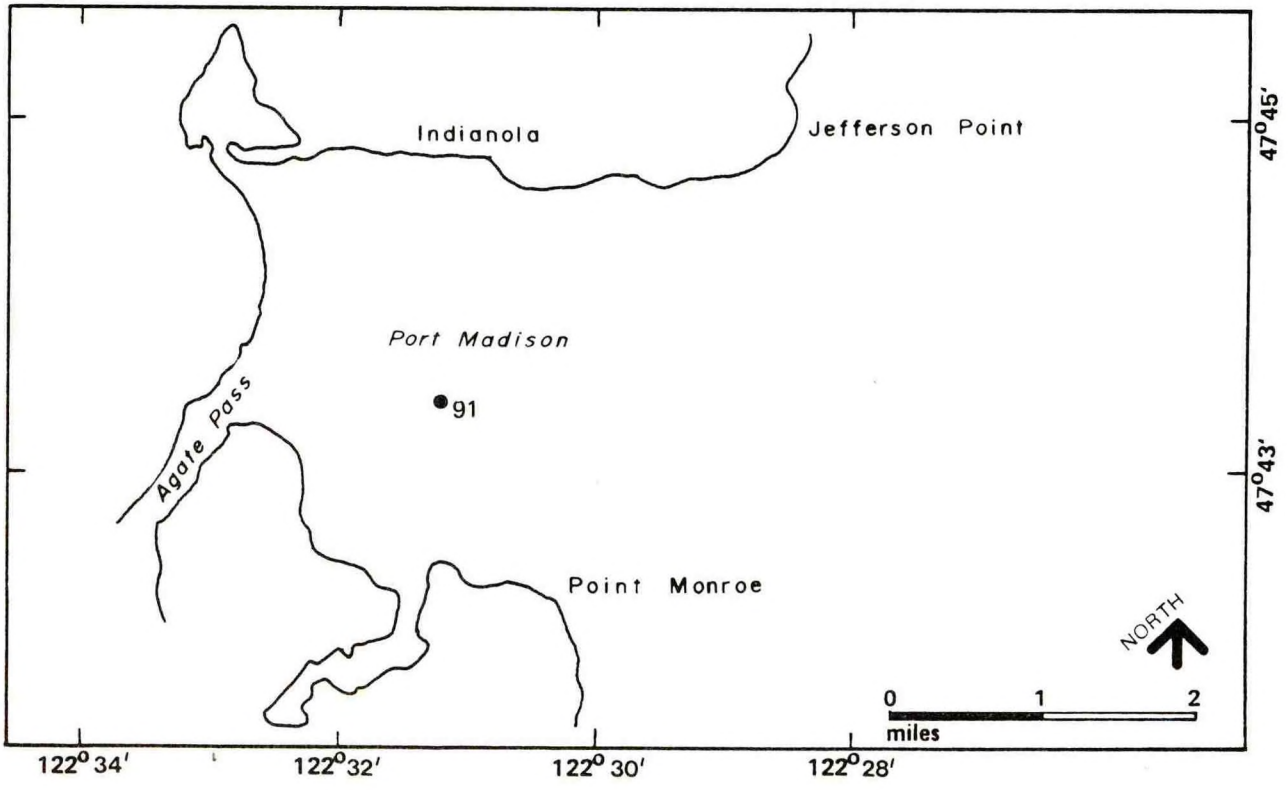


Figure 7. Station Location in Port Madison.

TABLE I

Toxicity Rating of Stations Tested Based on Previous Work by Chapman et al. (1982)

Station	Individual Ratings[a] Lethality	Respiration	Genotoxicity	Comments
ELLIOTT BAY				
2	0	0	++	only genotoxic effects
4	+	++	++	highly toxic
12	0	++	0	only respiration effects
15	0	++	++	high toxicity
17	0	0	++	only genotoxic effects
21	0	++	0	only respiration effects
26	0	0	+	low genotoxicity
29	0	++	++	high toxicity
37	0	++	++	high toxicity
COMMENCEMENT BAY				
42	0	0	0	no apparent toxicity
47	0	++	++	high toxicity
49	0	+	++	high toxicity
52	0	++	++	high toxicity
57	0	++	++	high toxicity
61	0	++	++	high toxicity
63	0	++	0	only respiration effects
67	0	++	++	high toxicity
70	0	++	++	high toxicity
71	0	++	0	only respiration effects
SINCLAIR INLET				
82	0	++	++	high toxicity
84	0	++	++	high toxicity
PORT MADISON				
91	0	0	+	low genotoxicity

[a]

Rating	Description
0	no significant effects
+	significant effects
++	highly significant effects

polychaete larvae, juveniles and adults; and, fish cells grown in vitro. The choice of particular tests and test species was mediated in part by the availability of organisms for testing, particularly within the time frame of this study which was conducted in autumn 1982. An account of the relative sensitivities of the tests and organisms is provided below.

2.3.1 Oyster Larvae

Toxicity tests with fertilized eggs of the Pacific oyster (Crassostrea gigas) have been used by numerous authors to evaluate environmental contaminant effects in water samples (e.g. Loosanoff and Davis, 1963; Dimick and Breeze, 1965; Woelke, 1967, 1972; Cummins, 1973, 1974; Carr et al., 1974; Schink et al., 1974; Carr, 1975; Cummins et al., 1976; Cardwell et al., 1977a, b; Bourne et al., 1981; Coglianese, 1982). Tests have also been done with sediment samples by Schink et al. (1974), Cummins (1973, 1974), Cummins et al. (1976) and Cardwell et al. (1977a).

The oyster or bivalve embryo bioassay technique is described in Standard Methods (APHA, 1980). This technique has proven to be a rapid and reliable indicator of environmental quality. Marine bivalve embryos and larvae are more sensitive to contaminants than the adult of the same species (Bryan, 1971; Calabrese et al., 1973; Hrs-Brenko et al., 1977).

During the first 48 h of embryonic development, fertilized oyster eggs normally develop into free-swimming, fully shelled veliger larvae. Both the failure of the eggs to survive and the proportion of larvae developing in an abnormal manner, are used as indicators of polluted conditions (Woelke, 1972; APHA, 1980). Both lethality and percentage abnormal larvae were measured in the present study.

2.3.2 Surf Smelt Eggs and Larvae

Surf smelt (Hypomesus pretiosus pretiosus) are intertidal spawners which serve as a food source for commercial species such as salmonids (Hart and McHugh, 1944; Hart, 1973) and are themselves of commercial and recreational importance (Pentilla, 1978; Levy, 1982). Effects of environmental contaminants in sediments on egg and larval stages may directly influence the spawning and rearing areas available for successful surf smelt reproduction. Any reduced survival of juveniles from these rearing areas has implications for the health of successive trophic levels.

Marine fish eggs have been utilized extensively for development and hatchability studies in relation to toxicants (e.g. Rosenthal and Alderdice, 1976; Misitano, 1977; McGreer and Munday, 1981; Reid and McGreer, 1982; Morgan et al., 1982). Previous studies with surf smelt in Puget Sound, conducted by the National Marine Fisheries Service, have involved the exposure of eggs and larvae to oil (Misitano, 1977, pers. comm.; Hawkins and Stehr, 1982; Malins et al., 1982; Weber, pers. comm.). Other investigators in Oregon have measured hatching success of surf smelt in relation to Mt. St. Helen's ash (Boehlert, pers. comm.)

There is a good data base available concerning the early life history and development of surf smelt (Schaefer, 1936; Yap-Chiangco, 1941), and surf smelt eggs are available throughout most of the year (May through December) (Pentilla, 1978). Effects of toxicants on surf smelt eggs and larvae may include: increased mortality, changes in the timing of hatching, and larval abnormalities (Malins et al., 1982). Testing in the present study looked at: hatching success, timing of development, appearance of abnormalities, and larval survival after hatching.

2.3.3 Polychaete Life-cycles

Capitella capitata is a cosmopolitan estuarine and marine polychaete indigenous to Puget Sound, which forms an important link in benthic trophic structure, and may comprise part of the diet of commercial fish stocks. It is often found in areas near domestic sewer outfalls and has been used as a biological indicator of organic enrichment by various investigators (Reish, 1955; Reish and Barnard, 1960; Warren, 1976; Pearson and Rosenberg, 1978; Petrich and Reish, 1979; Reish, 1978, 1980). The susceptibility of C. capitata to toxicants changes at different stages in its life-cycle, and toxicants can affect both growth and reproduction (Reish, 1980). Adult C. capitata are less tolerant than other polychaetes to particular chemical contaminants (Reish, 1978) and the larvae are more sensitive than adults to most toxicants (in some cases LC50 values are over an order of magnitude lower) (Reish, 1980). Thus, although C. capitata is often abundant in environmentally degraded areas of Puget Sound, the viability of individuals found in areas with high contaminant levels may be reduced.

C. capitata has been used in a variety of pollutant studies related to environmental contaminant levels (Reish, 1978, 1980; Reish et al., 1974; Reish and Carr, 1978), and can be cultured through a full generation within a few weeks. Methodologies for conducting life-cycle studies with C. capitata are well documented in the literature and are described in Standard Methods (APHA, 1980). Effects of toxicants during life-cycle testing may include: increased mortality, suppression of reproduction, and induction of larval abnormalities (Reish, 1980; Reish et al., 1974, 1976). Testing in the present study looked at: survival at all life-cycle stages, any abnormalities, growth rate, and timing to reproduction.

2.3.4 Cell Reproduction

Previous studies have shown that rainbow trout gonad cells (RTG-2) grown in vitro demonstrate genotoxic and cytotoxic effects in the presence of both known mutagens such as benzo(a)pyrene (B(a)P), and some Puget Sound sediment extracts (Kocan et al., 1979, 1982; Chapman et al., 1982). These previous studies suggested that, at least in some instances, toxic effects were due to the presence of a mixed function oxidase (MFO) enzyme system in the trout cells which is capable of metabolizing otherwise inert xenobiotics to active toxic substances.

Extensive research has been conducted into the MFO system by numerous investigators. Two excellent reviews on the MFO systems of aquatic fish and invertebrates are provided by Chambers and Yarbrough (1976) and Stegeman (1981), who note that aquatic animals are capable of much the same metabolic processing of xenobiotics as mammals (i.e. microsomal oxidation, reduction and conjugation). Exposure of fishes to compounds such as B(a)P and aflotoxin B_1 has induced tumor formation that is directly attributable to the processing of these compounds by the MFO system. Marine invertebrates also possess this metabolic capability, which can often be induced by prior exposure to such MFO inducing agents as benzanthracene (Nereis virens) or contaminated sediment (Capitella capitata). Tests for MFO activity in copepods, oysters and clams have thus far given inconsistent results.

In the present study the sublethal effects of organic sediment extracts were measured in relation to cellular proliferation. Two fish cell lines with very different MFO activity were chosen for testing: bluegill fry cells (BF-2) and rainbow trout gonad cells (RTG-2). The bluegill cells have little or no MFO activity while the rainbow trout cells have high levels of this enzyme (Diamond and Clark, 1970; Clark and Diamond, 1971). The two cell lines were exposed in vitro to a series of increasing concentrations of Puget Sound sediment extracts during their logarithmic growth phase. Calculation of the rate of cell proliferation during extract exposure allowed determination of effects on cell growth and reproduction. Differences between the two cell lines allowed detection of sediments containing direct acting toxins requiring no metabolic activation as well as indirect or pro-toxins that require conversion to a toxic form.

The use of fish cell cultures allowed observation of toxic effects directly on cells by eliminating the influence of the integrated defense mechanisms found in the intact animal (e.g. excretion, detoxification, storage, non-specific binding). As such, these tests provided an extremely sensitive direct test of sediment toxicity. Testing in the present study looked at both inhibition of cell proliferation and lethality (cytotoxicity).

2.4 Sediment Collection

Sediment samples were collected using a 0.1 m^2 van Veen grab modified with top screens and rubber flaps to minimize surface sediment disturbance. Opening of the screens allowed access to the surface of the material in the grab without disturbing the contents. Five grabs were collected at each station.

Sediments were removed from the grab by carefully inserting the barrel of a commercial aluminum cookie press (approximately 5 cm diameter) into the sediment without disturbing the sample. Holes had been bored in the barrel 6 cm above the end of the press to allow the overlying water to escape and to ensure uniform penetration. Each grab was sub-cored seven times (approximately 120 ml of sediment per sub-core).

The sub-cores from all van Veen grabs at each station (7 x 5 = 35) were extruded into a single polyethylene bag and carefully homogenized to yield approximately 4 L (6 Kg wet weight) of sample. Aliquots for possible future analyses were then transferred to solvent-rinsed, 120 ml glass jars with teflon cap liners using a stainless steel spatula. Aliquots for cell reproduction testing were similarly transferred to solvent rinsed, 475 ml glass jars with teflon cap liners. The remainder of the sample was sealed in the polyethylene bag.

All samples were frozen within 24 h of collection, stored with dry ice during transport, and kept frozen prior to analysis. Subsamples for chemical analysis were delivered to the National Marine Fisheries Service (Montlake Laboratory) for storage.

2.5 Toxicity Testing

2.5.1 Oyster Larvae

Adult Pacific oysters (age 3-4 years) were obtained from Baynes Sound Oyster, a commercial oyster farm located in Union Bay, British Columbia. Stock oysters were held at ambient seawater temperatures (13-15°C) in clean flowing seawater prior to conditioning and testing. All oysters were fed plankton suspensions ad libidum.

Groups of oysters were removed as required for testing, cleaned of fouling organisms and placed in continuous-flow conditioning trays to permit gonadal maturation. The oysters were thermally conditioned (19±1°C) for 3 weeks, with individual oysters periodically sacrificed to determine the state of gonadal development.

Following maturation, two oysters of opposite sex were placed together in a shallow pyrex spawning dish filled with filtered, UV-treated seawater at 20°C. The spawning dish was then placed in a water bath (30°C) and 1.5 h later approximately 20 ml of a prepared sperm suspension from a sacrificed male oyster was added. After 1 h the water in the spawning dish was replaced with fresh 20°C treated seawater, and the dish placed back into the 30°C water bath. Spawning of the male occurred soon afterwards, followed by the female. After 30 min, the adult oysters were removed from the spawning dish. The fertilized eggs were washed through a Nitex screen (250 μm) to remove excess gonadal tissue, and were then suspended in 2.5 L of treated seawater at 20°C. Fertilized eggs were examined microscopically for the formation of polar bodies. When polar bodies were observed, egg density was determined from triplicate counts of the number of eggs in 1 ml samples of a 1:99 dilution of homogeneous egg suspension.

Sediment bioassays were conducted using the general oyster embryo bioassay technique of Woelke (1972), modified for use with sediments. Testing was conducted in clean (rinsed with 5% nitric acid) one liter Nalgene polyethylene bottles. Fifteen grams (wet weight) of the appropriate sediment were added to each bottle and the volume

brought up to 750 ml with treated seawater to make a final concentration in all test containers of 20 g (wet weight) of sediment per liter seawater. Two controls were prepared and run concurrently. One control contained the same concentration of clean sediment (from off West Beach, Whidbey Island, a collection site for the sensitive amphipod Rhepoxynius abronius). The other control contained clean seawater. All containers were run in duplicate (22 test sediments + 2 controls x 2 = 48 containers).

Testing was initially conducted following the methods of Cardwell et al. (1977a) which involved adding developing eggs to the containers then sealing them and rotating at 4 rpm for the 48 h test period. However, the physical effects of the suspended sediment and of rotation were such that few live larvae (less than 5%) were recovered, even from the controls. Thus, the procedure was modified and repeated as follows. The sediments were suspended by rotating at 10 rpm for 3 h, following which period the fertilized eggs were added and the suspended sediments allowed to settle. No additional agitation was provided.

Within 2 h of fertilization each container was innoculated with some 28,000 developing oyster embryos, to give an approximate concentration of 35/ml. The innoculated cultures were covered with paper towelling and air-incubated for 48 h at 20±1°C. After 48 h, the contents of each container were carefully poured through a Nytex mesh screen (42 µm) without disturbing the settled sediment, thereby retaining and concentrating the surviving oyster larvae (larvae caught in the sediments were invariably dead). The concentrated larvae were then washed into a 100 ml graduated cylinder to measure the volume of each sample, transferred to screw-cap glass vials, and preserved with 3% neutral formalin. Preserved samples (equal in volume to that containing 300-400 larvae in controls) were placed in Sedgewick-Rafter cells and examined at 100X magnification.

Normal and abnormal larvae were enumerated to determine percent survival and percent abnormalities. All larvae that failed to transform to the fully shelled, hinged, "D" shaped veliger were considered abnormal.

Salinity, dissolved oxygen and pH levels were initially adjusted in each container to 25 ppt, 8.0 mg/l and 8.0, respectively. These parameters were measured for each container at the termination of the bioassay.

2.5.2 Surf Smelt Eggs and Larvae

Adult surf smelt (Hypomesus pretiosus pretiosus) were collected by beach seine from the southwestern beaches (Spanish Banks area) of Burrard Inlet, B.C. on August 30, September 14, 16, 22, 29 and October 4, 1982. Sampling was conducted from high tide through ebb in late afternoon and into evening, a period when spawning adults were most abundant (Schaefer, 1936; Pentilla, 1978; Levy, 1982).

Sufficient numbers of gravid females were not obtained September 14, 22 and 29. Adults collected August 30 were used for preliminary

tests of spawning and incubation methods. Initial methodology involved spawning the adults into egg trays at the collection site, then transporting the fertilized eggs to the laboratory in oxygenated seawater. However, egg viability was very low with this method. Best egg viability was obtained by transporting adult surf smelt to the laboratory in 50 L plastic buckets filled with oxygenated seawater from the collection site and spawning them immediately upon arrival. This method was used for all test series. Clean dilution water for spawning and testing was obtained from Burrard Inlet.

Preliminary experiments were conducted to establish the procedure for exposing fertilized eggs to sediments. Initially the technique of Stanley (1977) was used, which involved allowing the eggs to attach to open egg trays then placing the trays onto test sediment. However, variations in the adhesive qualities of the eggs resulted in losses from these trays and closed containers were developed which, with a netting bottom, also allowed more intimate association with the sediment.

Surf smelt eggs require a daily cycle of water immersion and exposure for best hatching success (Misitano, 1977). The best diurnal combination of immersion:air drying was found, by preliminary experiment, to be 4 h immersion to 20 h of air exposure. This cycle was maintained in all test series.

Test procedures were as follows. In the laboratory, the sexual products from gravid males and females were gently expressed (by squeezing) over clean plexiglass trays containing plastic egg receptacles. These receptacles were constructed from plastic pipe (3 cm long, 5 cm outside diameter), the bottom of which was covered with 0.5 mm Nitex mesh screen. The receptacles were left in the trays for 1-2 h following spawning to ensure that fertilization had occurred and were then removed and used for testing.

Egg receptacles were individually fitted into a length of plastic pipe and held in place by friction, allowing easy removal and replacement for observation of egg development. The other end of the pipe was sealed with a plug into which a section of nylon rod was inserted. The whole assembly was suspended inside a 4 L wide-mouth glass jar (pre-cleaned with acid) fitted with a lid and half filled with clean seawater. The nylon rod was fitted through a hole in the lid and was held in place by cotter pins in one of two positions: with the egg receptacle immersed just above the bottom of the jar in contact with the sediment, or with the receptacle completely out of the water.

Each jar was fitted with a plastic air line attached to a perforated ring of air tubing at the bottom of the jar which allowed both complete oxygenation of the water and suspension of sediments prior to egg receptacle immersion. During immersion the air was turned off to prevent physical damage to the eggs. Complete oxygenation prior to immersion ensured that oxygen levels remained high during immersion.

Each jar was filled with a concentration of the appropriate sediment equal to 20 g (wet weight) per liter of seawater. Concentrations were prepared from frozen sediments immediately prior to testing. Controls were prepared and run concurrently. One control contained the same concentration of clean sediment (from off West Beach, Whidbey Island), the other control contained clean seawater. Two separate test series were run without duplication, using the fertilized eggs collected on September 16 (Series A) and October 4 (Series B).

Every effort was made to deposit similar numbers of eggs in all containers, however a scarcity of gravid females (only 2 of 6 fish collected) for Series A resulted in low numbers of eggs in some receptacles. This variability was somewhat reduced for Series B when six females were obtained, however inequalities were unavoidable. Egg receptacles were placed in jars with sediment and the exposure:immersion cycle was initiated.

Eggs were incubated at 11.5±1.5°C under a 12 h light/dark cycle in a controlled environment room. Water salinities were 14±2 ppt, the salinity of the collection site. Light control included 2 h of graduated dawn/dusk and simulated moonlight.

Reference receptacles were used concurrently with each test series. References consisted of receptacles suspended in aquaria with either clean seawater or clean sediment. At least one reference receptacle was examined daily to measure egg development and the results were used to determine the time intervals for examination of the test receptacles, thus minimizing possible handling stress.

Developmental observations were made with a Wild M5A dissecting microscope by removing egg receptacles from jars, and placing them directly under the microscope in a petri dish filled with clean seawater. Observations were made at the following developmental stages described by Yap-Chiangco (1941): 1. aggregation/migration of protoplasmic material leaving a clean perivitelline space; 2. optic development; 3. embryo two to three times full circle generally exhibiting movement and with pigmentation on the tail and yolk sac; 4. appearance of a dark polarized cap towards the later developmental stages; and, 5. hatching of larvae.

When larvae were first detected in a test series, daily observations were made of all receptacles. Live larvae were removed from receptacles by pipette and were carefully placed in pre-cleaned 150 ml wide-mouth jars containing the same concentration of freshly prepared sediment/water slurry as the respective incubation jars. The sediment was allowed to settle prior to larval introduction when oxygen levels were at saturation. After the first larvae were added, the jars were disturbed as little as possible to prevent physical damage to the larvae. High oxygen levels were maintained by periodic gentle aeration as necessary.

Attempts were made to feed the larvae with concentrated marine plankton (63-250 μm size range) collected by screening Burrard Inlet

seawater. Live larvae were visible in the jars when the jars were illuminated from behind. Dead larvae became opaque and sank into the sediment. Larvae were maintained for a minimum of 10 d from the day of first hatch, and survival was measured over this period.

2.5.3 Polychaete Life-Cycles

Trochophore larvae and adults of the marine polychaete worm Capitella capitata were purchased as isogenic strains from Dr. D. Reish (University of California, Long Beach). Testing was initiated with trochophore larvae, which were either obtained directly from Dr. Reish (received within 24 h of hatching) or by dissecting out the egg cases of mature females. No difference in response was noted between these two groups of trochophores. Trochophores and adults were all acclimated to the test conditions when received (20°C, 35 ppt salinity, 12 h light/dark cycle) and testing was initiated as soon as possible following receipt.

Testing was conducted using the general methodology described by Reish (1980) and APHA Standard Methods (1980), modified for use with sediments. Testing was conducted in clean (rinsed with 5% nitric acid), sterile 100 x 20 mm glass petri dishes with lids and involved the use of either sediment elutriates or whole sediments.

Sediment elutriates were prepared by placing 10 g (wet weight) of the appropriate frozen sediment in a 1 L acid-cleaned glass jar, and adding 500 ml of clean seawater (35 ppt salinity) to make a final concentration of 20 g (wet weight) of sediment per liter of seawater. The jars were shaken vigorously to suspend the sediments and ensure complete mixing, and sediments were allowed to settle for 3 h. Fifty ml of elutriate were then withdrawn by clean individual pipette from the upper water layer and added directly to the test containers for the bioassays. Sediment tests were conducted by adding a 2 mm layer of sediment to the bottom of each petri dish following elutriate water addition for an approximate sediment/water concentration of 500,000 mg/L (=ppm).

Testing was initially conducted in 3.78 L jars with aeration, however this method required periodic decanting of the water and removal of the worms for observation, resulting in physical damage and stress. The use of petri dishes, which was confirmed for full life-cycle studies in preliminary testing, allowed for constant observation of the worms with minimal disturbance.

Three separate experimental series were completed without replication. An initial series of elutriate tests was conducted with four sediment samples (Stations 61, 70, 84, 91) selected at random. Full series elutriate tests were conducted several weeks later with all sediment samples and a total of four controls (2 seawater controls, 2 controls with clean sediment elutriates using Whidbey Island sediment as per the oyster larvae and surf smelt bioassays). Full series sediment tests were conducted shortly after this with all sediment samples and a clean sediment control.

Twenty free-swimming trochophore larvae were randomly pipetted into each test dish along with a food suspension consisting of finely ground Tetramin and Enteromorpha (a green alga) in seawater. The dishes were then covered and kept at 20±1°C under a 12 h light/dark regime in a controlled environment room.

Elutriate cultures were fed twice weekly ad libidum. The amount of food was determined by the amount remaining from the previous feeding and was increased as the worms grew. Feeding schedules for sediment cultures followed those of the elutriate containers.

Solutions were changed in the elutriate cultures on a regular basis and replaced with fresh elutriate made with recently un-frozen sediment. The first change was made at 5 d after initiation when most of the larvae had settled; changes were then made at 3 and 4 d intervals for the duration of testing. Solutions were not changed in the sediment cultures, however once during the test period the top water layer (approximately 5 ml) was siphoned off and replaced with an equal amount of fresh elutriate. This procedure was followed to remove sediment-related accumulations of debris in the surface of some containers which could have interfered with surface air exchange.

Elutriates were changed by using 5 ml disposable pipettes connected to a peristaltic pump to remove all but 5 ml of solution from each container. Microscopic examination during pumping ensured that no individuals were removed with the old solution. Freshly prepared elutriate was added immediately following pumping.

Estimates of growth and mortality were made 2 d after initiation and then prior to every elutriate change, by placing each petri dish on a numbered 1 cm square grid and examining the bottom systematically under a Wild M5A dissecting microscope. Direct observations were only possible for elutriate tests and included estimates of larvae and/or juveniles present, observations of any abnormalities and, during early growth stages, measurements of the lengths of individual worms. During late growth stages, examination of the cultures concentrated on egg production and laying. When trochophore larvae were noted in the female tube, the tube was gently dissected under magnification for enumeration of eggs and larvae, and examination of eggs and larvae for abnormalities.

Sediment cultures were observed on an opportunistic basis during the course of the testing. After settlement of the larvae it became almost impossible to see the worms which burrowed into the sediment, hence observations of worms in particular test sediments were all made after termination when worms were sorted out under a dissecting microscope.

Initial elutriate tests (4 sediments) were terminated after 60 d; the second (full series) tests were terminated after 50 d at which time trochophores were detected in the control vessels. Sediment tests were terminated after 35 d at which time percent survival, mean length, number of segments per worm, and developmental stages (i.e., females bearing eggs) were determined.

2.5.4 Cell Reproduction

2.5.4.1 Preparation of Sediment Extracts

Sediment samples were frozen and stored until just prior to extraction. Each sample was then thawed, rehomogenized by careful but thorough stirring, and an aliquot (approximately 20 g wet weight) transferred to a clean tared beaker. This aliquot was dried to constant weight (80°C), dessicated and reweighed to determine the percent water. A second aliquot (approximately 150 g wet weight) was transferred to a tared, solvent-cleaned, 315 ml stainless steel centrifuge bottle with a teflon-lined screw cap, and weighed. The sample was then serially extracted with pesticide-grade solvents using the procedure of Malins et al. (1980) as previously used by Chapman et al. (1982), and summarized below.

Methanol (50 ml) was added to each centrifuge bottle, which was tightly capped and shaken vigorously for 2 min followed by centrifugation at 2000 rpm for 5 min. The clear solvent was decanted into a 1 L separatory funnel. The procedure was repeated twice more and the methanol extracts were combined in the separatory funnel, which was then closed and covered with aluminum foil.

One hundred ml of a dichloromethane/methanol (2:1 v/v) solution were added to the centrifuge bottle, the cap closed tightly and the bottle was shaken vigorously for two minutes to insure complete mixing. The bottle was then placed in a shaker table overnight (approximately 18 h), following which the sediment was settled by centrifugation at 2000 rpm for 5 min and the solvent decanted into the separatory funnel with the methanol. A second 100 ml aliquot of the dichloromethane/methanol (2:1) was added, the bottle shaken vigorously and placed on the shaker table for 6 h. The sediments were again settled with centrifugation and the solvents decanted.

The remaining sample was shaken vigorously for 2 min with approximately 30 ml of dichloromethane; it was centrifuged, and the solvent decanted into the separatory funnel. Another 100 ml of dichloromethane was added to the bottle, the cap secured, the bottle shaken vigorously, and placed on the shaker table overnight. The sediments were again settled with centrifugation and the solvent was decanted into the separatory funnel. A final 30 ml rinse of dichloromethane was added and the bottle shaken vigorously, followed by centrifugation and decanting. The sediment was then discarded.

Approximately 500 ml of cleaned, distilled water were added to the combined solvents in the separatory funnel. The funnel was carefully swirled and inverted (with frequent venting) for 2 min. The liquid phases were allowed to separate and the dichloromethane (lower) layer was drained into a 500 ml separatory funnel. The aqueous layer was re-extracted twice with 20 ml of dichloromethane and the remainder discarded. The dichloromethane fractions were combined in the 50 ml funnel and transferred, with rinsing, back to the 1 L funnel and

re-extracted with another 500 ml of distilled water. The dichloromethane was drained into the 500 ml funnel and the aqueous layer was extracted once more with 20 ml of dichloromethane. The latter solvent was added to the 500 ml funnel and the aqueous layer was discarded.

The dichloromethane was drained from the 500 ml separatory funnel through approximately 20 g of combusted and washed anhydrous sodium sulfate that was held in a 30 ml glass conical centrifuge tube with the tip cut off. The effluent from this mini-column was discharged into a 500 ml Kuderna-Danish flask with a 15 ml receiver. When empty, the 500 ml separatory funnel was rinsed with 20 ml of dichloromethane which was drained through the sodium sulfate column into the flask. The column was washed a final time with 10 ml of dichloromethane which was also drained into the flask.

Boiling chips were added to the Kuderna-Danish flask and a 3-ball Snyder column was placed on top. The solvent volume was reduced to about 5 ml on a hot water bath. When cooled, the sides of the flask were rinsed into the receiver with dichloromethane. The receiver was removed and the contents were quantitatively transferred to a tared conical centrifuge tube with a ground glass stopper. The sample was then taken almost to dryness on the hot water bath, followed by storage in a dessicator wrapped in aluminum foil with the stopper open slightly, until a constant weight was achieved upon reweighing the tube. This weight was the amount of extractable organic material.

After weighing, the tube was closed and wrapped fully in aluminum foil ready for cell reproduction testing. Extracts were treated with 1 ml of spectrophotometric grade DMSO for 24 h with frequent stirring on a vortex mixer. The DMSO was then removed to a glass vial and used as "stock" solution. Since not all extracted material was dissolved in the DMSO during testing, the centrifuge tubes were dried and reweighed to estimate the amount actually used in testing (fraction soluble). Both stock and extract solutions were stored in the dark under nitrogen until applied to the cell cultures.

2.5.4.2 Testing

Rainbow trout gonad cells (RTG-2) and bluegill fry cells (BF-2) were cultured in Leibovitz L-15 medium, pH 7.1 to 7.3, supplemented with 10% fetal calf serum and antibiotics. The cells were seeded into Corning 2 cm^2 multiwell plates at densities of 20,000 (RTG-2) and 90,000 (BF-2) cells per well and incubated at 18°C and 25°C, respectively. After the cells had settled and attached, six concentrations (dilutions) of each extract were prepared in duplicate in the culture medium and added to the cell cultures. These dilutions were based on total organic weight of the samples (1, 5, 10, 25, 50, 75 µg/ml) and were later corrected to reflect the concentrations of soluble extract by determining the tube weight prior to and after the final DMSO extraction.

Final extract concentrations were determined by first exposing cell cultures to a wide range of extract concentrations to determine the highest non-toxic dose which permitted cell proliferation. This high dose was then used to make all subsequent dilutions to which the cultures would ultimately be exposed. Since the preliminary dose range was based on the total organic content of each sediment extract, the final dose to which the cells were exposed had to be calculated from the percent of total organics which were soluble in the final DMSO solvent.

$$\text{Final extract conc. } (\mu g/ml) = \frac{((\text{total extract wt.}) - (\text{insoluble extract wt.})) \times \text{dilution factor}}{\text{ml of culture media}}$$

Due to the diversity of sediment types, each sample had a different solubility in DMSO, thus accounting for the slight differences seen in the concentration ranges listed in Table 8.

Control cultures consisted of six replicates of untreated cells, six replicates of solvent (0.5% DMSO) treated cells, two replicates of an extraction blank which consisted of residues of all of the solvents used during sediment extraction, and six replicates of 0.5 µg/ml B(a)P treated cells for comparison with a known toxicant. In previous anaphase aberration testing (Chapman et al., 1982) a 0.25 µg/ml B(a)P concentration caused chromosomal damage; a higher B(a)P concentration was used in the present study to significantly reduce cell numbers. From these controls, determinations were made of normal levels of cell growth, the effect, if any, of the solvent, and verification of the presence or absence of an MFO system.

After a 96 h exposure period, the cultures were rinsed once with buffered saline (pH 7.1) to remove any unattached dead cells. The remaining live cells were removed for counting by the addition of 0.01% EDTA/Trypsin to each culture well. The total number of cells in each replicate well was determined using a Coulter electronic particle counter (Model ZBI).

Mean number of cells for each treatment was compared to the mean control values (96 h controls = 100%) and any concentration which reduced the final cell count by 20% (equivalent to approximately 2 standard deviations of the mean of the controls and thus representing 95% confidence limits) or more was considered inhibitory. When cell numbers at the end of 96 h were below the starting cell number, that concentration of extract was considered cytotoxic (i.e. produced cell death).

3.0 RESULTS

3.1 Sediment Characteristics

The sediment characteristics determined while preparing the extracts for cell testing, the percent water and extractable organic matter, are summarized in Table 2 along with the fractions of the extractable matter which dissolved in the DMSO used for cell testing. As in the previous study (Chapman et al., 1982), higher percent water and

TABLE 2

Sediment Physical and Chemical Parameters

Station Number	Water %	Extractables[a] %	Soluble[b] %
2+[c]	23.4	0.023	84
4 *[c]	42.4	0.567	56
12	47.1	0.424	81
15+	28.1	0.072	63
17+	24.4	0.018	92
21 *	52.0	0.436	51
26+	50.5	0.961	54
29+	50.7	0.483	59
37	45.1	0.140	39
42+	50.6	0.395	57
47 *	55.2	0.635	35
49	69.2	0.903	36
52+	64.7	0.935	39
57+	42.5	0.167	87
61+	50.9	0.187	43
63+	42.1	0.159	67
67+	43.6	0.108	85
70+	56.3	1.872	89
71+	41.0	0.091	48
82+	58.2	0.305	53
84+	45.3	0.155	55
91+	37.5	0.063	74

a. Organic matter recovered from the sediments by dichloromethane/methanol extraction, expressed as percent of dry weight sediments.

b. Quantity of the extracted material which was soluble in DMSO, expressed as percent of extractable organic matter (extractables).

c. A plus (+) notation after a station number indicates that live organisms were observed in the sediments during sampling. A star (*) indicates that a bad odor, e.g. H_2S, was noted in the sample during collection.

higher organic matter content were observed in nearshore, protected areas, where finer sediments accumulate, in comparison with more exposed stations. Also shown in Table 2 are incidental observations made during sample collections of the presence of live organisms in the sediments and of sediment odor. Living organisms, particularly annelids and small bivalves, were observed in the majority of sediment samples. No special effort was made during the cruises to look for organisms; only readily obvious biota were recorded. As a result, the remaining stations may have had viable organisms which were not observed. Only a few of the samples had a readily apparent odor, usually from H_2S: Station 4, near the Denny Way CSO in Elliott Bay; Station 21, from the Duwamish West Waterway; and, Station 47 in the Hylebos Waterway, Commencement Bay. No organisms were noted in any of these three stations.

3.2 Toxicity Testing

3.2.1 Oyster Larvae

The results of the Pacific oyster embryo bioassays are summarized in Table 3. In addition to larval numbers, oyster embryo response is expressed in terms of mean percent abnormal larvae, and mean percent relative survival (compared to controls). Salinity, pH and dissolved oxygen values determined at the termination of the bioassay are provided in Appendix B. These parameters remained at acceptable levels in most test cultures including those eliciting adverse larval response: salinity range 25-25.1 ppt; pH range 7.6-8.0; dissolved oxygen range 6.3-6.6 mg/l in controls, 5.0-6.1 mg/l in test sediments except Station 52. Station 52 showed slightly depressed oxygen values, to 4.3 mg/l.

The Pacific oyster embryos used in the bioassays were of excellent quality, as indicated by the low percentage of abnormal larvae in the control seawater (1.1%) and control sediment (1.6%) cultures. These values are well below the 3% abnormality rate suggested by Woelke (1972) as acceptable for oyster larvae bioassay controls.

Sediment samples gave dramatic differences in responses ranging from extremely toxic to non-toxic. Station 52 was the most toxic to developing oyster embryos; no live larvae were found after 48 h. The percentage of abnormal larvae exhibited by oyster embryos exposed to sediments from Stations 4, 21, 26, 29, 37, 42, 47, 49, 57, 61, 70 and 82 exceeded the single sample marine water quality criterion of 20% larval abnormality, proposed by Woelke (1972). The following additional stations exceeded Woelke's (1972) proposed multiple sample quality criterion of 5% larval abnormality: 15, 63, 71 and 84. Stations 2, 12, 17, 67 and 91 all had less than 5% larval abnormalities.

The survival values generally agreed with the data on abnormalities. For the 12 stations with greater than 20% larval abnormalities, mean relative survival was low, with a range of 2-46%. Of the 4 stations with between 20% and 5% larval abnormalities, only Station 15 had a mean survival greater than 55% (88%) which, coupled with a low 6%

TABLE 3

Oyster Larvae Bioassay Data

Station	Replicate	Total Larvae	Normal Larvae Total	Normal Larvae Percent	Abnormal Larvae Total	Abnormal Larvae Percent	Mean Values: Number of Larvae	Mean Values: Percent Abnormal	Mean Values: Percent Relative Survival[a]
2	A	172	162	94	10	6	286	3	78
	B	400	391	98	9	2			
4	A	51	14	27	37	73	107	30	30
	B	164	136	83	28	17			
12	A	376	368	98	8	2	350	2	96
	B	324	315	97	9	3			
15	A	408	403	99	5	1	322	6	88
	B	236	200	85	36	15			
17	A	350	340	97	10	3	351	2	96
	B	352	345	98	7	2			
21	A	7	3	43	4	57	9	78	2.5
	B	11	1	9	10	91			
26	A	23	8	35	15	65	20	50	6
	B	17	12	71	5	29			
29	A	86	12	14	74	86	47	86	13
	B	8	1	12	7	88			
37	A	32	20	62	12	38	26	31	7
	B	20	16	80	4	20			
42	A	13	1	8	12	92	22	91	6
	B	32	3	9	29	91			

Table 3 Cont'd

Station	Replicate	Total Larvae	Normal Larvae Total	Normal Larvae Percent	Abnormal Larvae Total	Abnormal Larvae Percent	Mean Values: Number of Larvae	Mean Values: Percent Abnormal	Mean Values: Percent Relative Survival[a]
47	A	32	7	22	25	78	46	42	13
	B	60	35	77	14	23			
49	A	64	44	69	20	31	84	24	23
	B	104	83	80	21	20			
52	A	0	0	-	0	-	0	-	0
	B	0	0	-	0	-			
57	A	110	67	70	33	30	88	32	24
	B	67	45	67	22	33			
61	A	81	56	69	25	31	166	22	46
	B	252	204	81	48	19			
63	A	97	82	85	15	15	105	15	29
	B	112	96	86	16	14			
67	A	351	333	95	18	5	272	4	75
	B	194	189	97	5	3			
70	A	8	0	0	8	100	8	94	2
	B	9	1	11	8	89			
71	A	162	153	94	9	6	200	13	55
	B	238	194	82	44	18			
82	A	79	38	48	41	52	78	45	22
	B	78	49	63	29	37			
84	A	97	80	82	17	18	74	18	20
	B	50	40	80	10	20			

Table 3 Cont'd

Station	Replicate	Total Larvae	Normal Larvae Total	Normal Larvae Percent	Abnormal Larvae Total	Abnormal Larvae Percent	Mean Values: Number of Larvae	Mean Values: Percent Abnormal	Mean Values: Percent Relative Survival[a]
91	A	173	165	95	8	5	167	4	46
	B	161	156	97	5	3			
Sediment Control	A	497	488	98	9	2	487	2	134
	B	477	470	99	7	1			
Seawater Control	A	355	351	99	4	1	364	1	100
	B	374	370	99	4	1			
Combined Sediment and Seawater Controls							426		
							($\bar{n}$=4;S.D.= 72; $\bar{X}$±2S.D.= 282-570)		

a. In terms of the seawater control which, following standard (Cummins, 1973, 1974; A.P.H.A., 1980) procedures, is assigned a survival value of 100%.

rate of larval abnormalities, indicates low toxicity at this station. The 5 stations with less than 5% abnormalities had generally good survival rates (75-90%) with the exception of Station 91 which had a mean relative survival rate of 46%, indicating high acute lethality at this station.

Based on the combined data for the oyster larvae bioassays, the following stations showed high toxicity: 4, 21, 26, 29, 37, 42, 47, 49, 52, 57, 61, 70 and 82. The following stations showed intermediate toxicity: 15, 63, 71, 84 and 91. The following stations appeared to be relatively non-toxic: 2, 12, 17 and 67.

3.2.2 Surf Smelt Eggs and Larvae

The results of the development studies with surf smelt eggs are summarized in Table 4 and data sheets are provided in Appendix C. Because many eggs were covered with a coating of fine sediment, it was not possible to accurately assess development in terms other than optic development, larval hatching and larval survival. A great deal of variability was observed precluding statistical analysis of the data, however a number of observations can be made concerning sediment-related effects.

Percent of larvae hatched in relation to fertilized eggs proved to be highly variable. Seawater and sediment control cultures had a combined overall mean hatching success of 14%, while test cultures ranged from 0-41%. These data suggest that hatching may have been enhanced in some sediments. Although this high variability makes it difficult to assess relative hatching success, three stations showed exceptionally low hatching success in both test series: Station 12 (2%), Station 26 (0%), and Station 52 (1%). In addition, premature hatching (larvae hatching before development was complete) was noted in Series A at the following stations: Station 29 (4 of 33 larvae; 12%); Station 49 (4 of 15 larvae; 27%); and, Station 57 (3 of 72 larvae; 4%).

In the first series the ratio of larvae hatched dead (but not prematurely) was higher for some test sediments (up to 100%) than for controls (5-20%) (Appendix D). However, the second series did not follow this pattern and the significance of this observation is unknown.

Timing of development was not significantly affected by any of the sediments. Time from fertilization to median larval hatch ranged in the first series from 19-28 d (a mean of 24 d) and in the second series from 18-30 d (a mean of 26 d). Similar increases in hatching time related to later spawning times have been noted between eggs collected in November and December (Malins et al., 1982).

The larvae were maintained after hatching in containers with test sediments, however they could not be induced to feed, an effect noted by other investigators (Yap-Chiangco, 1941; Stanley, 1977). Consequently possible variations in growth related to sediment exposure could not be assessed.

TABLE 4

Surf Smelt Development Data

Station	Replicate	Total Eggs	Optic Development Total	Optic Development Percent[a]	Larvae Hatched Total	Larvae Hatched Percent[a]	10d Larvae Survival Total	10d Larvae Survival Percent[b]	Mean Values: Total Eggs	Optic Development (%)[a]	Larvae Hatch (%)[a]	10 d Larvae Survival (%)[b]
2	A	205	0	0	0	0	0	0	205	12	8	0
	B	205	50	24	34	17	0	0				
4	A	116	75	65	13	11	6	46	162	29	7	25
	B	208	20	10	11	5	0	0				
12	A	88	0	0	0	0	0	0	89	3	2	0
	B	90	5	6	3	3	0	0				
15	A	70	6	9	5	7	2	40	151	10	7	10
	B	232	25	11	16	7	0	0				
17	A	118	0	0	0	0	0	0	167	15	10	0
	B	216	51	24	34	16	0	0				
21	A	58	16	28	16	28	0	0	122	60	22	0
	B	186	130	70	37	20	0	0				
26	A	70	2	3	0	0	0	0	141	17	0	0
	B	212	45	21	0	0	0	0				
29*	A	182	70	38	61	33	16	26	155	23	20	20
	B	128	0	0	0	0	0	0				
37	A	120	12	10	1	1	0	0	158	18	8	0
	B	196	45	23	23	12	0	0				
42	A	122	5	4	2	2	0	0	151	18	7	9
	B	180	50	28	20	11	2	10				
47	A	32	3	9	3	9	0	0	102	43	19	0
	B	172	85	49	35	20	0	0				
49*	A	215	50	23	32	15	21	66	208	23	10	53
	B	202	45	22	8	4	0	0				
52	A	29	2	7	0	0	0	0	98	2	1	0
	B	166	2	1	1	1	0	0				
57*	A	138	72	52	72	52	2	3	144	39	32	2
	B	150	40	27	19	13	0	0				
61	A	99	70	71	61	62	49	80	96	58	41	62
	B	92	40	43	18	20	0	0				

Table 4 Cont'd

Station	Replicate	Total Eggs	Optic Development Total	Optic Development Percent[a]	Larvae Hatched Total	Larvae Hatched Percent[a]	10d Larvae Survival Total	10d Larvae Survival Percent[b]	Mean Values: Total Eggs	Optic Development (%)[a]	Larvae Hatch (%)[a]	10 d Larvae Survival (%)[b]
63	A	235	95	40	56	24	11	19	156	37	30	12
	B	78	60	77	38	49	0	0				
67	A	148	15	10	5	3	0	0	175	21	9	0
	B	202	60	30	27	13	0	0				
70	A	69	13	19	13	19	6	46	124	34	25	13
	B	178	70	39	49	28	2	7				
71	A	240	2	1	0	0	0	0	245	32	16	0
	B	250	155	62	77	31	0	0				
82	A	168	90	54	57	34	16	28	163	46	24	21
	B	158	60	38	21	13	0	0				
84	A	81	20	25	17	21	2	12	154	28	6	11
	B	226	65	29	2	1	0	0				
91	A	112	35	31	35	31	24	69	119	16	16	65
	B	126	4	3	2	2	0	0				
Sediment Control	A	148	55	37	10	7	7	70	153	29	4	58
	B	158	35	22	2	1	0	0				
Seawater Control	A	136	95	70	59	43	48	81	127	43	27	70
	B	118	15	13	10	8	0	0				
Combined Sediment and Seawater Controls									140	36	14	68

a. Calculated based on total eggs (e.g. for Station 4, % optic development = $\frac{75 + 20}{116 + 208}$ = 29%)

b. Calculated based on total larval hatch (e.g. for Station 4, % larvae survival = $\frac{6 + 0}{13 + 11}$ = 25%)

* Stations showing premature larvae hatching in Series A.

Despite the fact that they did not feed, larval survival over 10 d was high in Series A controls both with sediment (70%) and with seawater alone (81%). In contrast, 11 Series A test sediments had no larvae alive after 10 d, and 8 test sediments had larvae survivals between 3 and 46%. Only three stations had survival rates comparable to the controls: Station 49 (66%), Station 61 (80%) and Station 91 (69%) in Series A. The results of Series B testing, in which larval survival was never more than 10%, suggest that the larvae hatched from this spawn were not viable for unknown reasons. The combined larval survival data from the two test series indicated that control level survivals were only found at Stations 49, 61 and 91.

Based on the developmental data obtained in this study with surf smelt eggs and larvae, sediments from the following stations showed evidence of toxic effects: 26 (no larvae hatched); 12 and 52 (low hatching success and no larval survival); 29 and 57 (low larval survival and premature hatching); 2, 4, 15, 17, 21, 37, 42, 47, 63, 67, 70, 71, 82 and 84 (low larval survival); and, 49 (premature hatching). Based on good hatching success and larval survival coupled with the absence of any apparent abnormalities, the following stations appear to be relatively non-toxic to surf smelt eggs and larvae: Stations 61 and 91.

3.2.3 Polychaete Life-Cycles

The results of the polychaete life-cycle bioassays are summarized in Table 5 for the second (full series) sediment elutriate tests, and in Table 6 for whole sediment tests. Data sheets are provided in Appendix D for all elutriate tests including the first series of 4 sediments. Salinity, pH and dissolved oxygen values determined during testing remained at acceptable levels (salinity, 35$\pm$1 ppt; pH, 7.8-8.0; dissolved oxygen, > 5 mg/l).

Two types of larval mortality were distinguished during the sediment elutriate testing: death before and during metamorphosis. Trochophores dying before metamorphosis disintegrated rapidly, those dying during metamorphosis were found on the bottom of the test containers partly transformed into juvenile worms. The latter type of larval mortality was noted for the following stations: 17, 21, 26, 42, 47, 70 and 84. All stations including controls showed some trochophore mortality, however only Stations 4, 17, 37 and 47 had significantly ($p=0.05$) higher larval mortalities than controls.

Growth of juvenile and adult worms was monitored twice during the sediment elutriate tests and once at termination of the whole sediment tests. Prior to sexual maturation (up to Day 16), growth of juvenile worms in elutriate sample dishes ranged from 0.17 to 0.39 mm/worm/d. Mean growth rate for controls were 0.23 mm/worm/d. Only one test sediment, Station 37, had a lower growth rate than controls (0.17). The only growth rate significantly ($p=0.05$) different from controls was Station 57 (0.39), which was almost twice as high.

TABLE 5

Capitella capitata Test Data for Sediment Elutriates

Station	Larvae Mortalities During Metamorphosis[a]	Larvae Survival[a] Total	%	Growth and Development: Growth Rate (mm/d) to Day 16	Growth Rate (mm/d) to Day 29	Days to First Observed Eggs Produced	Days to First Observed Eggs Laid	Overall Survival to Day 26: Total	Overall Survival to Day 26: Percent
2	0	17	85	0.32	0.31	19	42	16	80
4	0	10	50	0.28	0.27	26	-[c]	10	50
12	0	16	80	0.28	0.27	23	34	15	75
15	0	11	55	0.32	0.27	26	-[c]	11	55
17	2	10	50	0.29	0.35	26	50	8	40
21	1	16	80	0.29	0.33	23	37	15	75
26	4	15	75	0.33	0.29	19	37	15	75
29	0	16	80	0.34	0.33	19	35	15	75
37	0	9	45	0.17	0.25	29	42	8	40
42[b]	2	14	74[b]	0.34	0.37	26	43	13	68[a]
47	2	10	50	0.25	0.28	26	35	10	50
49	0	14	70	0.36	0.32	26	-[c]	14	70
52	0	11	55	0.25	0.26	34	48	11	55
57[b]	0	15	79[b]	0.39	0.33	19	50	15	79[a]
61	0	14	70	0.31	-[c]	19	-[c]	14	70
63	0	18	90	0.34	0.29	23	-[c]	18	90
67	0	16	80	0.28	0.33	19	34	12	60
70	2	14	70	0.35	0.40	23	-[c]	13	65
71	0	12	60	0.28	0.33	19	40	12	60
82	0	14	70	0.29	0.33	19	44	14	70
84	2	14	70	0.32	-[c]	19	-[c]	13	65
91	0	18	90	0.33	0.32	19	45	17	85

Table 5 Cont'd

Station	Larvae Mortalities During Metamorphosis[a]	Larvae Survival[a] Total	Larvae Survival[a] %	Growth and Development: Growth Rate (mm/d) to Day 16	Growth Rate (mm/d) to Day 29	Days to First Observed Eggs Produced	Days to First Observed Eggs Laid	Overall Survival to Day 26: Total	Overall Survival to Day 26: Percent
Sediment Control A	0	16	80	0.23	0.34	29	42	14	70
Sediment Control B	0	17	85	0.26	0.23	34	43	16	80
Seawater Control A	0	15	75	0.17	0.26	26	-[b]	14	70
Seawater Control B	0	14	70	0.25	0.30	29	-[b]	13	65
Combined Sediment and Seawater Control Mean	0	4.5	22.5	0.23	0.28	29.5	42.5	14	71
S.D.	0	(±1.3)	(±6.5)	(±0.04)	(±0.05)	(±3.3)	-	(±1)	(±6)

a. Tests initiated with n = 20 trochophore larvae
b. n=19 not 20; one trochophore lost during elutriate change
c. No data; worms died from undetermined causes

TABLE 6

Capitella capitata Test Data for Whole Sediments

Station	Survival[a] Total	%	Mean Length (mm) ± S.D.	Mean No. of Segments ± S.D.	Number of Females with Eggs[a]
2	9	45	8.3 ± 3.1	43 ± 8	2
4	12	60	6.1 ± 1.1	43 ± 7	2
12	11	55	5.6 ± 2.0	38 ± 8	3
15	10	50	6.5 ± 2.0	41 ± 9	3
17	11	55	8.1 ± 1.4	43 ± 4	3
21	1[b]	5	-	-	-
26	10	50	5.1 ± 2.2	38 ± 9	2
29	15	75	6.0 ± 2.3	38 ± 7	2
37	11	55	5.4 ± 1.3	38 ± 7	2
42	14	70	5.6 ± 1.5	39 ± 5	2
47	6	30	6.3 ± 1.8	42 ± 7	2
49	9	45	4.1 ± 1.6	-[c]	1
52	5	25	3.8 ± 1.6	32 ± 11	0
57	7	35	5.6 ± 2.1	38 ± 10	1
61	12	60	5.5 ± 2.3	42 ± 7	1
63	0	0	-	-	-
67	11	55	4.1 ± 1.8	36 ± 7	1
70	7	35	4.3 ± 1.1	34 ± 5	0
71	11	55	7.1 ± 2.3	39 ± 8	2
82	12	60	4.0 ± 0.7	32 ± 6	0
84	12	60	7.3 ± 2.3	48 ± 11	3
91	11	55	6.6 ± 1.6	42 ± 7	3
Control	16	80	7.1 ± 2.1	38 ± 8	5

a. On day 35; 20 trochophore larvae originally seeded in each.

b. Two live fragments (an anterior and posterior end) of 16 and 22 segments were found; the anterior portion subsequently survived.

c. Worms died and deteriorated rapidly following termination; segment counts were not made.

During the period of active egg development in maturing female worms (Day 29), measurements of length indicated that growth rates for controls had increased to an overall value of 0.28 mm/worm/d with test samples ranging from 0.25 to 0.40 mm/worm/d. None of the tests showed significantly (p=0.05) different growth rates from controls at this point.

The growth of C. capitata in whole sediments (Table 6) was roughly half that observed in the sediment elutriate samples (mean growth rate was 0.16±0.04 mm/worm/d for sediments compared to 0.31±0.05 mm/worm/d for elutriates). The reason for this difference is unknown.

Both mean length and mean number of segments were measured at termination of the sediment tests (Day 35). Counting number of segments did not provide a good indication of growth as very small worms were found to have a similar number of segments as much larger worms (Table 6). Length measures were much more effective in distinguishing size differences. Controls had a mean length of 7.1±2.1 mm with only two test sediments having values above 8 mm: Station 2 (8.3 mm) and Station 17 (8.1 mm). Worms exposed to sediments from eight stations had mean lengths significantly (p=0.05) less than controls indicating slower growth: Stations 26, 37, 42, 49, 52, 67, 70, 82.

Mature females with developed internal egg masses were observed in all sediment elutriates. Egg masses were observed in individual test containers from 19 to 34 d after initiation (Appendix D). Slowest egg production was noted for controls (26-34 d), Station 37 (29 d) and Station 52 (34 d).

Unfortunately, an unknown pathogen unrelated to the tests killed all worms in 10 of the 16 containers (including both seawater controls) between Day 23 and Day 50, all but one test group dying before eggs were laid. Thus a complete comparison of timing to egg laying cannot be made. Of the remaining test containers, in all cases except Station 17, at least one set of eggs was laid prior to termination of testing on Day 50. Results from the first elutriate test series (Appendix D) demonstrated that C. capitata raised in elutriates from Stations 61, 70 and 82 (which were all killed in the second series) produced and laid eggs. Eggs were first laid in controls 42 d after initiation. Time to egg laying for test containers ranged from 34 to 50 d with worms exposed to sediments from Stations 52 (48 d) and 57 (50 d) taking the longest to lay eggs. It thus appears that although egg production and laying may be delayed or speeded up by test sediments, total inhibition of reproduction is rare.

Of the whole sediment samples from which more than one worm was recovered at termination (Day 35), only three (Stations 52, 70, 82) did not contain mature females bearing eggs (Table 6). One female recovered from Station 84 was actually laying eggs on Day 35 while two females from Station 17 laid eggs three days after being removed from the sediment. Females recovered from the whole sediment samples were sexually mature at a much smaller size than females exposed to sediment elutriates.

The number of eggs laid varied from 20 to 200+ (Appendix D). Although there were indications that the total number of eggs laid could be affected by test sediments (the controls laid 100+ egg masses), the data are insufficient to draw definite conclusions.

Abnormal bifurcated larvae of the type documented by Reish et al. (1974) were only noted on one occasion: one trochophore out of a mass of 166 eggs laid in the first series in Station 84 sediment elutriate was bilobed anteriorly. This abnormality rate was 0.6% and was not considered significant. A few misshapen eggs were noted in some test containers (Appendix D) but the numbers were not large and the significance of this observation is unknown.

Few mortalities occurred after larval settlement and overall survival showed the same pattern as that of the larvae. Overall survival in sediment elutriates from Stations 4, 17, 37 and 47 was significantly lower than controls.

Survival of C. capitata was generally lower in whole sediments (range of 0-80%) than in elutriates (range of 40-90%). Best survival in sediments was noted in the control, and Stations 29 and 42 (70-80%). Intermediate survival (45-60%) was noted for Stations 2, 4, 12, 15, 17, 26, 37, 49, 61, 67, 71, 82, 84 and 91. Low survival (0-35%) was noted for Stations 21, 47, 52, 57, 63 and 70.

Based on the life-cycle data for C. capitata obtained in this study, sediments from the following stations showed evidence of toxic effects: Stations 26, 42 and 84 (abnormal larval metamorphosis and significantly slower growth in sediment tests); Stations 4 and 63 (significant sediment or elutriate mortalities); Station 37 (significant elutriate mortalities and slower growth in sediment tests); Stations 49 and 67 (significantly slower growth in sediment tests); Station 21 (abnormal larval metamorphosis and low survival in sediment tests); Station 57 (slow egg laying in elutriate tests and low survival in sediment tests); Station 82 (slow egg production in sediment tests); Station 17 (abnormal larval metamorphosis, significant elutriate mortalities and slow egg laying in elutriate tests); Station 47 (abnormal larval metamorphosis, and significant mortalities in both elutriate and sediment tests); Station 52 (slow egg laying in elutriate tests, slow egg production in sediment tests, low survival and significantly slower growth in sediment tests); and, Station 70 (abnormal larval metamorphosis; low survival, slow egg production and significantly slower growth in sediment tests). Stations 2, 12, 15, 29, 61, 71 and 91 appeared to be relatively non-toxic with Station 29 showing significantly higher early growth than any other tests including controls.

3.2.4 Cell Reproduction

Table 7 summarizes the results from control cultures for cell replication. Both cell lines showed an approximately 150% increase in numbers over 96 h. Neither the DMSO solvent nor the extraction blank affected cell proliferation. B(a)P had no effect on the BF-2

TABLE 7

Summary of Cell Proliferation in Control Cultures

Cell Line	Number of Cells at Start	Number of Cells After 96 h (% increase)			
		Untreated Control	DMSO Control	B(a)P Control	Extraction Blank
		n=6	n=6	n=6	n=2
RTG-2	20,000	49,100[a] (145%)	50,300[a] (151%)	31,800 (59%)	45,400 (127%)
BF-2	90,000	240,000[b] (167%)	252,000[b] (180%)	246,000 (173%)	242,000 (169%)

[a] Standard Deviation (S.D.) = 4,100
[b] Standard Deviation (S.D.) = 30,000 (derived from untreated and DMSO controls)

cultures, but did reduce proliferation of the RTG-2 cells by approximately 60%. This result indicates the importance of an active MFO system in metabolizing otherwise inert compounds such as B(a)P to form toxic products.

Table 8 summarizes the data obtained from exposure of cells to sediment extracts from 22 stations. BF-2 cultures showed little response to any extract; significant decreases in cell numbers were only observed at five stations (17, 57, 63, 67, 91) and only at the highest extract concentration tested. In contrast, RTG-2 cultures exhibited reductions in cell numbers relative to controls at all stations and showed typical dose response to increasing concentrations of sediment extract. The range of concentrations tested at each site were similar but not identical, hence a relative toxicity rating is provided in Table 8 based on ranges of extract concentrations which produced a significant (P=0.05) inhibition of proliferation.

Only one station, 57, exhibited effects at all concentrations tested. Eight other stations (2, 12, 17, 26, 29, 37, 61, 84) showed effects at extract concentrations below 10.0 µg/ml; seven stations (15, 21, 47, 49, 63, 82, 91) showed effects at concentrations between 10 and 19.9 µg/ml; and, six stations (4, 42, 52, 67, 70, 71) only showed effects at concentrations of 20 µg/ml or more. Extracts from nine stations also produced cytotoxicity (the number of cells was reduced during testing) in RTG-2 cells (Stations 2, 17, 21, 42, 57, 61, 63, 84, 91).

In both RTG-2 and BF-2 cultures a stimulatory effect on cell growth was noted at concentrations from all stations which were not inhibitory or toxic. This unexplained phenomenon is frequently observed in biological test systems treated with organic compounds and is not well understood. The phenomenon may be analogous to the rapid growth stimulating properties produced by some herbicides.

4.0 DISCUSSION

4.1 Oyster Larvae Toxicity Tests

The object of these tests was to determine the effects of various Puget Sound sediment samples on the development and survival of Pacific oyster embryos (Crassostrea gigas). This species was selected for testing due to its wide distribution in Puget Sound, its importance as a commercial fishery, the sensitivity of the larvae, and previous extensive use of the test technique in Puget Sound.

The value of using oyster embryo bioassays to detect toxicity in contaminated sediments has been confirmed by a variety of authors (Schink et al., 1974; Cummins, 1973, 1974; Cummins et al., 1976; Cardwell et al., 1977a). The development of larval abnormalities is used as an indication of chemical toxicity in combination with mortality. However, mortalities (but not abnormalities) can also be caused by such factors as low salinity and the presence of dinoflagellates (Cardwell et al., 1977b).

TABLE 8

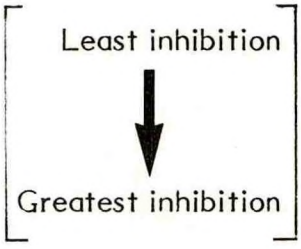
Effects of Puget Sound Sediment Extracts on Proliferation of Rainbow Trout Gonad (RTG-2) and Bluegill Fry (BF-2) Cells in vitro

Station	Absolute Concentrations Tested (µg/ml)[b]						Relative Toxicity[a]	
							RTG-2	BF-2
2	0.84	4.2	8.4	21	42	63*	3	0
4	0.56	2.8	5.6	14	28	42[c]	1	0
12	0.81	4.0	8.1	20	40	61	3	0
15	0.63	3.1	6.3	16	32	47	2	0
17	0.92	4.6	9.2	23	46	69*	4	1
21	0.51	2.5	5.1	13	26	38*	2	0
26	0.53	2.7	5.3	13	27	40	4	0
29	0.59	2.9	5.9	15	30	44	4	0
37	0.39	1.9	3.9	9.7	20	29	4	0
42	0.57	2.8	5.7	14	29	43*	1	0
47	0.35	1.8	3.5	8.8	18	26	2	0
49	0.36	1.8	3.6	9.0	15	27	2	0
52	0.39	1.9	3.9	9.7	20	29	1	0
57	0.87	4.4	8.7	22	44	65*	5	1
61	0.43	2.2	4.3	11	22	32*	4	0
63	0.67	3.4	6.7	17	34	50*	2	1
67	0.85	4.3	8.5	21	43	64	1	1
70	0.89	4.5	8.9	22	45	67	1	0
71	0.48	2.4	4.8	12	24	36	1	0
82	0.53	2.7	5.3	13	27	40	2	0
84	0.55	2.8	5.5	14	28	41*	4	0
91	0.73	3.7	7.3	18	37	55*	2	1

Table 8 Cont'd

[a] Toxic concentrations at which cell proliferation was inhibited by sediment extracts were scored on the following scale:

0 = no effect
1 = significant at 20 µg/ml and above
2 = significant from 10 - 19.9 µg/ml
3 = significant from 5 - 9.9 µg/ml
4 = significant from 1 - 4.9 µg/ml
5 = significant at less than 1 µg/ml.



[b] n=2 replicates per concentration

[c] Dashed underlining indicates concentrations that were inhibitory to RTG-2 cells; inhibition of BF-2 cells only occured at the highest single concentration tested.

* Indicates concentrations that were cytotoxic (resulted in cell death).

The data collected during this study indicated that 13 of the 22 stations tested were highly toxic to oyster larvae, 5 stations were less toxic, and 4 were relatively non-toxic. Chemical toxicity, as defined by the development of significant larval abnormalities, was indicated for those stations showing a positive response with the following possible exceptions. Station 91 had a low level of abnormalities (4%, within the accepted range for no toxicity) but a survival rate of only 46%. No abnormality levels were determined for Station 52 as no larvae survived the testing, and this station had the lowest dissolved oxygen values at termination.

The results of the present study compare well with previous sediment tests with oyster larvae in the Duwamish River. Cummins (1973) found that the response of oyster embryos to Duwamish River samples from the area of our Stations 21, 26 and 37 was characterized by greater than 20% abnormal larvae at sediment concentrations of 10 g/L (wet weight). In the present study, larval abnormalities at these stations ranged from 31-50%. Schink et al. (1974) reported concentrations of 0.5-1.5 g/L (wet weight) of Duwamish River sediment composites resulted in 100% abnormal development. In the present study a maximum abnormality rate of 86% was noted for the Duwamish, at Station 29.

In evaluating these results it must be remembered that oyster larvae are planktonic not demersal, hence this test was used mainly as an indication of toxic sediment effects. However, previous work (Chapman et al., 1982) has shown that toxicity is not restricted to the sediments but was also observed, at some sites, in samples of the overlying waters. Hence, it cannot be assumed that oyster larvae will be unaffected by contaminated sediments in the natural environment. Cardwell (1979) notes that the larvae of other Puget Sound bivalves (i.e. Manila littlenecks, Venerupis japonica; Olympia oyster, Ostrea lurida) are as sensitive as the Pacific oyster. Thus, the present test results suggest cause for concern related to stations showing toxicity in the present test.

4.2 Surf Smelt Egg and Larvae Toxicity Tests

Surf smelt are intertidal spawners on open to semi protected beaches. Adhesive eggs are deposited in the intertidal zone between +7 to +11 feet above MLLW, protected from heavy surf and dessication (Schaefer, 1936; Hart et al., 1944). Surf smelt are commercially and ecologically important in Puget Sound and spawn over more extended time periods than other marine fish. Maximum numbers of spawners are usually observed in June and July then decline (Levy, 1982), hence the difficulties encountered by the present study in obtaining sufficient spawners in September and October for testing. The tests performed with surf smelt eggs were used as a measure of relative sediment toxicity to marine fish eggs during development.

The low hatching success observed in control and most test sediments in the present study was not unexpected. Experiments were begun immediately after eggs were fertilized in the laboratory and hatching success in controls averaged 14%. Misitano (1977) found that his

attempts to strip adults and fertilize eggs in the laboratory resulted in a very low percentage fertilized and an almost total loss of viable eggs to fungus growth. Because of Misitano's (1977) work, toxicity studies conducted by National Marine Fisheries Service (NMFS) using surf smelt from Puget Sound have involved the collection of naturally deposited eggs from known spawning areas and microscopic examination prior to test initiation to ensure viability (Weber, NMFS, Seattle, WA, pers. comm.). The average time interval between fertilization and the use of the eggs in experiments by NMFS is 4 d, however even with this delay to ensure that live eggs are selected for testing, hatching success in controls is only 49±3% (Hawkins and Stehr, 1982; Malins et al., 1982). Under natural conditions, where fertilization appears to be virtually 100% successful, less than 10% of the eggs survive to hatching (Pentilla, 1978). The related American smelt (Osmerus mordax) has a mean survival to hatching of 1% (Rupp, 1965).

Hatching appeared to be enhanced with exposure to particular sediments (Stations 57, 61 and 63 in particular), which may be due to either the effects of low toxicant levels, or to physical stimulation of the eggs from attached sediment particles. Malins et al. (1982) noted that low hydrocarbon concentrations accelerated hatching over controls, and Misitano (1977) noted that physical stimulation enhanced hatching. Both possibilities may have occurred as Station 61 exhibited no apparent toxic effects to eggs or larvae while Stations 57 and 63 showed effects on the larvae.

The period of development observed in the present study (up to 30 d from fertilization to hatching) was consistent with other reports. Hatching optimally takes between 8 and 30 d depending on water and air temperature (Yap-Chiangco, 1941; Pentilla, 1978), increasing towards the end of the spawning season, and can take up to 36 d (Malins et al., 1982).

Previous work with surf smelt (Malins et al., 1982) has shown the following effects occur with exposure to crude oil: reduced growth inside the egg, arrested eye development, diffuse pigment prior to hatching, reduced hatching success, and reduced larval survival. Due to the fact that most eggs were coated with fine sediments during this study, observations could not be made of development (growth, onset of pigmentation) inside the egg. However, no evidence of arrested eye development was noted in hatched larvae. Both reduced hatching success and reduced larval survival were noted for particular sediments. More subtle damage to the developmental stages (i.e. necrotic tissue damage to eye and brain cells as observed by Hawkins and Stehr, 1982 with exposure of surf smelt larvae to crude oil) may have occurred, but was not determined in the present study.

Exposure to three sediments (Stations 29, 49, 57) resulted in premature hatching of eggs. Premature hatching related to toxicant exposure (PCB's, DDT, cadmium) has been observed with coho salmon (Oncorhynchus kisutch), pike (Esox lucius) and herring (Clupea harengus pallasi) (Halter and Johnson, 1974; Rosenthal and Sperling, 1974; Westernhagen et al., 1975). A possible explanation for this response

is an alteration of the egg surface due to toxicants, decreasing the permeability of the chorion to oxygen and thus inducing early hatching (Halter and Johnson, 1974). Reduced permeability may also be responsible for egg mortality prior to hatching.

Survival of non-feeding surf smelt larvae is related directly to the amount of yolk reserve; starved larvae hatched in the laboratory from summer spawnings can survive up to 26 d with a mean survival of 18 d (Stanley, 1977). The larvae from the first test series (Series A, spawned September 16) exhibited good control survival and differential survival in the test sediments. In contrast, the eggs of the second test series (Series B), which took slightly longer to hatch and were spawned a month later (October 4), died very rapidly in control and test containers. This difference between the two series may reflect differences in yolk reserve, or differences between the health and age of spawning adults used for the two series, or some other factor. Kilambi and DeLacey (1967) have shown that discrete populations of surf smelt can exist in the same area while Stanley (1977) has shown that surf smelt produce different sized eggs at different ages. For instance, 2-year old fish have eggs some 50% heavier than 1-year olds. No attempt was made in the present study to age the fish used for spawning nor to evaluate differences between eggs obtained from different spawnings.

National Marine Fisheries Services personnel have noted that surf smelt larvae exposed to adverse levels of toxicants are unable to maintain their equilibrium in the water column and sink to the bottom of test containers (Weber, NMFS, Seattle, WA, pers. comm.). This effect was also noted in the present study. In control containers and those showing low larval mortalities, the larvae remained in the water column; in the other containers the larvae sank to the bottom where they quickly died.

The data obtained during this study indicated that 20 of the 22 stations tested showed some evidence of toxicity to surf smelt development stages. Only Stations 61 and 91 showed no apparent toxic effects. In evaluating these results, it must be remembered that surf smelt are intertidal spawners hence spawning would not normally occur in proximity to the subtidal sediments tested herein. However, the use of this species serves as an indication of potential effects to marine fish eggs and larvae and suggests that long-term developmental effects may be a feature of the majority of the tested sites. The relative tolerance of surf smelt compared to important demersal spawning species such as herring and cod has not been determined. However the present results were obtained with only 4 h of daily direct exposure to the sediments. In addition, although surf smelt do not presently spawn in Elliott Bay, Commencement Bay or Port Madison, significant surf smelt spawning beaches are located in Sinclair Inlet (Dexter et al., 1981) and are vulnerable to possible toxicant release from sediments.

4.3 Polychaete Life-Cycle Tests

Capitella capitata are indigenous to Puget Sound and are common in areas showing high levels of chemical contamination (Malins et al.,

1980). Previous studies attempting to determine contaminant-related effects on this species have examined natural populations for gross and microscopic lesions with negative results (Malins et al., 1980). In contrast, data from the present study indicated that 3 of the 22 stations tested were highly toxic to C. capitata during some stage of its life-cycle and 12 were moderately toxic.

Despite abundant evidence for both acute and sub-lethal toxicity, C. capitata appeared able to grow and reproduce successfully in all tested sediments. A positive enhancement was observed in some sediments after settlement, which may partly explain why this species is abundant in contaminated sediments. Capitella capitata appear to respond to concentrations of readily assimilated organic matter (Grassle, 1980) and in the present study better growth and development were noted in many silty test sediments compared to seawater alone or to sandy control sediments.

Capitella capitata has been shown to comprise an assemblage of at least 6 sibling species (Grassle and Grassle, 1976). Type I was tested in the present study, conforming to almost all previous toxicity testing with C. capitata. As noted in this study, Type I are extremely opportunistic, capable of increasing rapidly in numbers, attaining large population size and early maturation despite high mortality (Grassle, 1980).

Present study results confirm previous observations that C. capitata larvae are more sensitive than are adults (Reish, 1980). Significant mortalities only occurred prior to completion of metamorphosis; few sediment-related deaths of juveniles and adults were noted. Induction of abnormal larval metamorphosis, noted for 7 sediments, has not been previously reported. Metamorphosis of C. capitata larvae is under hormonal control (Bhup and Marsden, 1982) and interference with these hormones may be responsible for the observed effects.

The present study results indicate the tested sediments can cause death, affect growth and delay reproduction. All of these effects have been previously demonstrated in laboratory tests with individual chemical contaminants such as metals (Reish et al., 1974; Reish, 1980). A previously reported contaminant-related effect, induction of abnormal larvae, was rare in the present study. However, abnormal larvae only appear in significant numbers (greater than 1%) in the second generation following continuous exposure to specific toxicants (Reish et al., 1974). The present study was terminated after production of the first generation hence the induction of abnormal larvae in later generations is unknown.

The period of development observed in the present study was generally consistent with other reports. Life-cycles for Type I C. capitata are generally completed in 30-40 d with eggs beginning to develop in the coelom after about 20 d. Some examples of longer development times were observed in this study.

The present results provide a comparison of elutriate and whole sediment toxicity tests, indicating generally good agreement.

However, some differences were noted. Stations 4 and 37 showed significant mortalities in elutriate but not in sediment tests, while high mortalities in Station 63 sediments were not noted in elutriates. Stations 82, 67 and 49 exhibited growth inhibition only in sediment tests. The reason for these differences is not known.

Type I C. capitata are probably not the only Type found in Puget Sound. Differences between Types include breeding period, presence and duration of a planktonic stage, and number of eggs. However, the results of the present study provide a good indication of the response of the species as a whole to tested sediments. In addition the present results indicate that C. capitata can survive and reproduce in all tested sediments, and provide data on the biological activity of various sediments based on the type and level of toxic response noted.

4.4 Cell Reproduction Tests

The data obtained during this study indicate that extracts of all of the sediments tested are capable of adversely affecting the reproductive capacity of fish cells exposed in vitro, provided that these cells possess an active mixed function oxidase (MFO) enzyme system. The BF-2 (bluegill) cells, which do not possess an active MFO system, were only affected by the highest concentrations of five sediment extracts. The high concentrations of organic material comprising these positive exposure levels (40-60 µg/ml), in the BF-2 cells suggests that nonspecific toxicity rather than inhibition of cellular reproduction is the cause. Previous studies with sediment extracts have shown that at or above 40-50 µg/ml of extract all sediments were toxic to fish cells (Chapman et al., 1982). Conversely, the obvious dose response to increasing concentrations of extract in the RTG-2 (Rainbow trout) cells suggests a true adverse but sublethal effect on cell proliferation. This effect is apparently the result of the conversion of otherwise inert compounds in the extract (e.g. polycyclic aromatic hydrocarbons) to toxic compounds by the MFO system in this cell line.

The difference in response between the two cell lines represents an excellent example of how two similar test systems can give very different results following exposure to the same compounds. It also suggests that the different responses of organisms to toxicants may be partly dependent on the presence or absence of specific enzyme systems. Similarly, differences in effects on different life-cycle stages or populations of the same species may also reflect the presence or absence of inducible enzyme systems.

The fact that cell proliferation was reduced or impaired by extracts from different sites does not indicate that the same compound(s) are present at each site. The effects observed could result from the presence of any number or combination of compounds which the cells are capable of metabolizing. Compounds such as PCB's and metals have not been shown to be metabolizable by the MFO system to a toxic form and were possibly diluted out of the extract while attempting to reach testable non-toxic concentrations.

The sublethal responses detected in the fish cells serve as sensitive direct tests of sediment toxicity. If translated to effects at the whole organism level, these responses might result in such subtle effects as low hatching weight, reduced growth, incompletely developed organs at the time of hatching, or a reduction in the number of viable gametes produced at spawning.

4.5 Combined Test Results

An overall ranking of the 22 stations based on reproductive impairment testing is provided in Table 9. The tested stations can be ranked in terms of toxicity as follows with combined ratings of 6 and above considered to indicate "high" toxicity, and values of 4 and less considered to indicate "low" toxicity:

High Toxicity

i. Station 57;
ii. Stations 17, 21, 26, 37, 42, 47, 70, 84;
iii. Stations 29, 63;
iv. Stations 4, 49, 52, 61, 82;

Low Toxicity

v. Stations 2, 67, 71, 91; and,
vi. Stations 12, 15.

The most toxic tested areas were Commencement Bay Waterways (Stations 42, 47, 49, 52, 57, 61, 63, 70) and the Duwamish River (Stations 21, 26, 29, 37). Stations in Elliott Bay were of variable toxicity, with Alki Point (Station 17) and Denny Way (Station 4) showing greatest toxicity and two waterfront stations (Stations 12, 15) showing least toxicity. Stations in Sinclair Inlet (Stations 82, 84) were of high toxicity while outer Commencement Bay (Station 71) showed low toxicity. The reference station (Station 91, Port Madison) was among the least toxic but did exhibit some effects.

The results of previous broad-scale toxicity testing at these stations is provided in Table 1. For the purposes of the following comparison, stations in Table 1 with 1-2 positives are considered to be of low toxicity while stations with 3-5 positives are considered to be of high toxicity.

Comparison of data from Chapman et al. (1982) with the present study results indicates a very high level of agreement. The following stations showed low toxicity in both studies: 2, 12, 71, 91. The following stations showed high toxicity in both studies: 4, 29, 37, 47, 49, 52, 57, 61, 70, 82 and 84. The additional testing conducted herein indicated that the following stations were more toxic than previously determined: 17, 21, 26 and 63.

Major differences between the two studies were only noted at three stations. Station 42 was found to be non-toxic by Chapman et al.

TABLE 9

OVERALL RATING OF TESTED STATIONS

The significance of each test is assumed to be equal; "0" = no effect, "+" = an effect. Summation of effects yields toxicity ratings.

	Oyster Larvae					Surf Smelt			
Station	>5% Abnormal	>20% Abnormal	48 h Survival <50%	No Survival	Rating (Number of Positives)	≤2% Hatching Success	10d Larval Survival <50%	Premature Hatching	Rating (Number of Positives)
2	0	0	0	0	0	0	+	0	1
4	+	+	+	0	3*	0	+	0	1
12	0	0	0	0	0	+[a]	0	0	1
15	+	0	0	0	1	0	+	0	1
17	0	0	0	0	0	0	+	0	1
21	+	+	+	0	3*	0	+	0	1
26	+	+	+	0	3*	+[a]	0	0	1
29	+	+	+	0	3*	0	+	+	2*
37	+	+	+	0	3*	0	+	0	1
42	+	+	+	0	3*	0	+	0	1
47	+	+	+	0	3*	0	+	0	1
49	+	+	+	0	3*	0	0	+	1
52	0	0	+	+	2*	+	0	0	1
57	+	+	+	0	3*	0	+	+	2*
61	+	+	+	0	3*	0	0	0	0
63	+	0	+	0	2	0	+	0	1
67	0	0	0	0	0	0	+	0	1
70	+	+	+	0	3*	0	+	0	1
71	+	0	+	0	2	0	+	0	1
82	+	+	+	0	3*	0	+	0	1
84	+	0	+	0	2	0	+	0	1
91	0	0	+	0	1	0	0	0	0

* Indicates most toxic station(s) for individual and combined tests

a. 0% survival

Table 9 Cont'd

Station	Cell Reproduction: Toxic to BF-2Cells[b]	Cell Reproduction: Cytotoxic to RTG-2 Cells[b]	Cell Reproduction: RTG-2 Cells Relative Toxicity 1-2[b]	Cell Reproduction: RTG-2 Cells Relative Toxicity 3-5[b]	Cell Reproduction: Rating (Number of Positives)	Capitella capitata: Significant Elutriate Mortality	Capitella capitata: Sediment Survival < 50%	Capitella capitata: Significantly Slower Growth Slow Egg Laying[c]	Capitella capitata: Abnormal Larval Metamorphosis[d]	Capitella capitata: Rating (Number of Positives)	Overall Rating (Number of Positives)
2	0	+	+	+	3*	0	0	0	0	0	4
4	0	0	+	0	1	+	0	0	0	1	6
12	0	0	+	+	2	0	0	0	0	0	3
15	0	0	+	0	1	0	0	0	0	0	3
17	+	+	+	+	4	+	0	+	+	3*	8
21	0	+	+	0	2	0	+	0	+	2	8
26	0	0	+	+	2	0	0	+	+	2	8
29	0	0	+	+	2	0	0	0	0	0	7
37	0	0	+	+	2	+	0	+	0	2	8
42	0	+	+	0	2	0	0	+	+	2	8
47	0	0	+	0	1	+	+	0	+	3*	8
49	0	0	+	0	1	0	0	+	0	1	6
52	0	0	+	0	1	0	+	+	0	2	6
57	+	+	+	+	4*	0	+	+	0	1	11*
61	0	+	+	+	3*	0	0	0	0	0	6
63	+	+	+	0	3*	0	+	0	0	1	7
67	+	0	+	0	2	0	0	+	0	1	4
70	0	0	+	0	1	0	+	+	+	3*	8
71	0	0	+	0	1	0	0	0	0	0	4
82	0	0	+	0	1	0	0	+	0	1	6
84	0	+	+	+	3*	0	0	+	+	2	8
91	+	+	+	0	3*	0	0	0	0	0	4

* Indicates most toxic station(s) for individual and combined tests

b. Toxic = reduction in cell reproduction; cytotoxic = reduction in cell numbers

c. Sediments and/or elutriates

d. Elutriates only

(1982), but was shown to have high toxicity in the present study. Conversely, Chapman et al. (1982) found that Stations 15 and 67 had high toxicity, while present study results indicated low toxicity.

Differences between the two studies may be partly explained by the fact that different samples were collected and tested for each study. Single grab samples were used by Chapman et al. (1982) in their broad-scale toxicity survey at 97 stations. The present study tested fewer (22) stations more intensely using composite grab samples to provide more representative responses from each station. Given the patchy nature of sediment toxicity (Swartz et al., 1982) and of sediment contaminants (Malins et al., 1980), it is not surprising that in some cases different grabs from the same station gave different results.

Another part of the reason for these differences lies with the different tests used. Chapman et al. (1982) found that Station 4 was the most toxic of all stations tested, based on the fact that it was the only one causing significant response in all three tests: mutagenicity, sublethal effects, and acute lethality bioassays. In the present study, Station 4 exhibited toxicity but, based on the particular series of sublethal tests employed, was not the most toxic. Similarly, stations considered in the present study to show high overall toxicity did not show high toxicity in each individual test used. For example, Station 57, considered the most toxic overall, did not show highest toxicity in C. capitata tests. The other fifteen stations with high overall toxicity showed little or no toxicity in at least one of the four individual tests. Station 2, which had low overall toxicity, showed high toxicity in cell reproduction tests. These results indicate both that different tests and organisms can have very different responses to the same sample, and emphasize the need for a series or progression of bioassay tests to properly determine the relative toxicity of field-collected sediment samples.

Despite variations in individual test results, the present overall toxicity assessments agree well not only with Chapman et al. (1982) but also with the only other published large-scale toxicity study in Puget Sound: Swartz et al.'s (1982) amphipod (Rhepoxynius abronius) bioassays in Commencement Bay and associated waterways. In particular, our Stations 47, 49 and 57 which are located in areas that Swartz et al. (1982) found to be lethal to R. abronius, show high toxicity in this study.

Not all biological effects noted in the present study were of an adverse nature. Positive effects (enhancement of growth, survival and hatching) were noted in cell reproduction, polychaete and surf smelt tests for particular sediments and developmental stages. Various investigators have shown that growth (e.g. coho salmon - McLeay and Brown, 1974), survival and hatchability (e.g. herring eggs - E.V.S. Consultants Ltd., 1977; Vigers et al., 1978) are enhanced by low toxicant concentrations. The positive enhancement demonstrated in this study may partly explain why certain species survive and apparently thrive in areas showing high toxicity. For instance, C. capitata were shown to suffer high larval mortalities in some

sediments but, following this, growth and development actually increased over controls among those that survived.

The present study results indicate that possession of an active MFO enzyme system capable of metabolizing xenobiotics serves not only for detoxification but can also render an organism more susceptible to toxic sediment effects presumably by the creation of toxic intermediates. These data represent an early step in our understanding of the physiological effects of toxicants in Puget Sound sediments on the biota. For instance, C. capitata has an active MFO system that takes several generations to activate and which can apparently respond to selective environmental pressures to increase tolerance to some toxicants (Lee and Singer, 1980). Flatfish, which show increased frequencies of histopathological disorders in areas of Puget Sound with highest toxic chemical concentrations (Long, 1982), have an active MFO system that is increased with exposure to such contaminants as oil and PCB's (Spies et al., 1982). However, the relevance of these data to populations in Puget Sound remains to be established.

The purpose of the present study was to conduct in-depth toxicity studies at 22 stations in order to verify the results of initial toxicity testing (Chapman et al., 1982), by using a broad suite of biological tests. As such, this study is not intended to include a comprehensive review and assessment of biological effects in the Sound. Other studies presently in progress will use chemical contaminant data now available for the majority of the 22 tested stations, and attempt to more precisely define the relationship of chemical contamination to biological effects.

The present study focused on both lethal and sublethal effects testing. Sublethal effects are particularly important because their ultimate effect may be a decreased fitness of exposed organisms resulting in diminished reproductive capabilities and increased susceptibility to predation and disease. In general, early developmental stages appear to be more sensitive to pollutants than juvenile and adult stages (Sprague, 1976). Thus the results of this study provide information relevant to precise definition of areas of concern in Puget Sound.

5.0 CONCLUSIONS

1. Tests involving the development of fertilized oyster eggs to larvae indicated that sediments from 18 of the 22 stations tested were capable of causing toxic effects (mortality and/or abnormalities).

2. Cell reproduction studies indicated that sediment extracts from all tested stations were capable of affecting cells (rainbow trout gonad) having an active mixed function oxidase (MFO) enzyme system capable of metabolizing xenobiotics, but that only 5 stations showed low level effects with cells not having an active MFO system (bluegill fry). These results may partially explain the different responses of different organisms or test systems exposed to the same sample.

3. Life-cycle studies with the polychaete worm Capitella capitata indicated that sediments (both elutriates and whole sediments) from 15 stations affected some life-cycle stage (i.e. mortality, abnormal larval metamorphosis, growth, timing of egg laying). Despite high initial larval mortalities in some samples, it appeared that C. capitata could complete its life-cycle in all sediments and both growth and reproduction were actually stimulated in the adult survivors of some contaminated sediments. These results fit well with this species' opportunistic nature which include the ability to increase populations rapidly despite high mortality.

4. Egg and larval studies with surf smelt indicated that sediments from 20 stations were capable of toxic effects (reduction in hatching success, reduction in larval survival and/or premature hatching).

5. There was good agreement between the results of the present study and of a previous broad-scale toxicity study (Chapman et al., 1982) which included these 22 stations. The few (3) exceptions were largely attributable to the use of different grab samples for testing and to the different tests used.

6. Based on the present study results, a subjective ranking system was used to categorize the relative toxicity of the 22 stations tested. The ranking, in decreasing order, is as follows:

   i. Station 57 (Blair Waterway);
   ii. Stations 17, 21, 26, 37, 42, 47, 70, 84 (Commencement Bay Waterways, Duwamish River, Alki Point, Sinclair Inlet);
   iii. Stations 29, 63 (Duwamish River, Sitcum Waterway);
   iv. Stations 4, 49, 52, 61, 82 (Denny Way CSO, Commencement Bay Waterways, Sinclair Inlet);
   v. Stations 2, 67, 71, 91 (Elliott Bay, Commencement Bay, Port Madison); and,
   vi. Stations 12, 15 (Elliott Bay).

7. The results of this study provide additional direct evidence linking specific geographic locations to reproductive effects.

6.0 RECOMMENDATIONS

A major recommendation remaining to be undertaken following the previous broad-scale toxicity study is to determine the sources of contamination related to specific toxic sites. This information is essential to allow mitigation of present adverse environmental effects in Puget Sound.

Specific additional recommendations arising from the present study are as follows:

1. The overall aereal extent of toxicity in the most toxic geographic areas (i.e. Hylebos and Blair Waterways, and the Duwamish River) needs to be determined. Such studies would serve to more accurately define the extent of toxic areas for possible clean-up.

2. Extensive toxicity tests have now been conducted with invertebrates, however the present surf smelt development tests represent one of the few tests conducted with commercially important fish species. The direct effects of contaminated sediments on commercially and ecologically important bottom-spawning fish species (i.e. herring, cod, flatfish) need to be determined to more completely assess the health of Puget Sound.

3. Detailed benthic infaunal studies should be conducted at selected tested stations to determine community structure in relation to toxicity data. Such information is essential to determine the ecological significance of sediment toxicity. Although previous benthic invertebrate studies have been conducted at some of these stations, the combination of small sample size, large screen size and incomplete taxonomic analysis precludes correlation of sediment toxicity to community structure.

4. All four different tests used in the present study provided useful information regarding sediment toxicity, however the surf smelt egg and larvae tests showed such high natural variability that we would not recommend them for further testing. The other three tests (oyster larvae, cell reproduction and polychaete life-cycle) are all recommended for future use in Puget Sound or elsewhere.

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