

Environmental Stresses and Fish Deformities in the Willamette River

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A. Introduction

The Willamette River was heavily polluted in the mid-20th century but recovered such that Hughes and Gammon (1987) stated, “there has been marked improvement in fish community quality in the Willamette River since 1945.” More recently, however, Portland Harbor was declared an EPA Superfund site, fish anomalies and parasite loads in agricultural areas of the basin were noted to be high (Wentz *et al* 1998; Waite and Carpenter 2000), and fish vertebral deformities were noted to be very high (Markle *et al.*, 2002). Oregon Department of Environmental Quality studies found northern pike minnow (*Ptychocheilus oregonensis*) in the Willamette River had skeletal deformities at rates of 1 to 74 % with the highest from river mile (RM) 27 to RM 55, an area known as Newberg Pool (Ellis *et al.* 2000). Skeletal deformity rates in unstressed fish populations were typically 2 to 5 % (Gill and Fisk 1966; Wells and Cowan 1982). The skeletal deformity rate in northern pike minnow from Newberg Pool was 75% in 1994, but 5% near Corvallis (RM 126) that same year (Ellis, 2000). Prevalence at the same site varied significantly between years. Skeletal deformity rates for juvenile northern pike minnow from the east bank of Newberg Pool were 52% in 1993 and 74% in 1994. There were also differences in lower Willamette River deformity rates between fish species (Markle *et al.*, 2002). Chiselmouth chub and northern pike minnow exhibited high rates of skeletal deformities (80 and 48%, respectively in 2000) while much lower rates occurred in bluegill sunfish and prickly sculpin (25 and 18%, respectively in 2000).

It has been suggested that skeletal deformities in fish are good bioindicators of pollution (Bengtsson 1979; Lemly 1997) and patterns of deformities can indicate if stress is chronic or acute. An increase in the frequency of deformities with age of fish (Slooff 1982) may suggest chronic stress, while acute stress may produce deformities at discrete life stages. (Lemly 1997). If skeletal deformation occurs at an early life stage, as appears to be the case with selenium, adults may appear healthy while the toxicity

impacts larvae (Lemly 1997). Skeletal deformities in fish are commonly attributed to pollutants, nutrition, genetics, or laboratory effects such as overcrowding. The mechanisms are seldom understood (Bengtsson 1979), but in most cases it appears in early development (Laale 1981). If prevalence of skeletal deformities serves as an environmental monitor, it is important to recognize that multiple stresses may contribute. Further, some developmental events may be particularly labile to specific stresses. Data from 2000 shows no differences in patterns of caudal deformities in Willamette River fishes between river miles 29 and 71 while deformities of the axial skeleton (vertebra) are substantially higher around RM 45-50, the Newberg Pool area (Fig. 1). Thus, it is important to categorize skeletal deformities, those which may indicate low-level stress (caudal deformities) and those which may indicate severe stress (axial skeletal deformities).

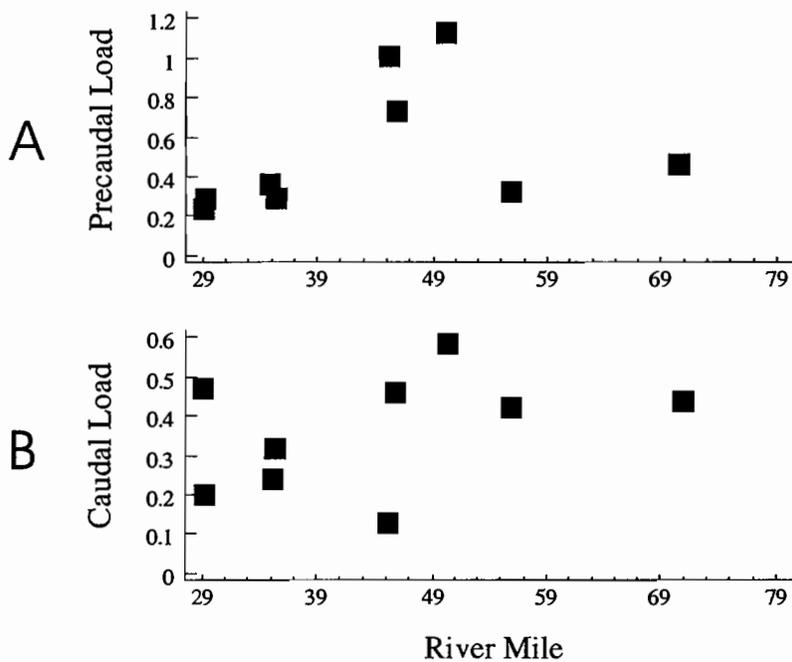


Figure 1. Comparison of axial skeleton deformity load (A) and caudal deformity load (B) by river mile in Willamette River fishes in 2000. The deformity load is the ratio of total lesions identified by x-ray divided by the total number of fish examined.

Newberg Pool is a rather slow, deep section of the lower river immediately upstream of Willamette Falls. Prevalence of axial skeletal deformities typically decreases with distance upstream of Newberg Pool. This project focuses on identification of environmental stresses that contribute to axial skeletal deformities in juvenile Willamette River fish. It considers common environmental variables such as temperature and pH, infectious agents, and pollutants including persistent bioaccumulative toxicants (PBTs), as factors that can increase axial skeletal deformity rates. The work will integrate information from the scientific literature, x-ray examination of fish from field and museum collections, histopathology, analyses of water physical and chemical conditions, fish tissue concentrations of PBTs, laboratory toxicity tests, and identification of infectious agents in lesions. Fractionation and bioassay of chemical constituents of water and suspended sediments from two sites in Newberg Pool and a reference site provides a novel approach to identifying specific chemical groups that contribute to deformities. Comparisons of results of field work on Newberg Pool and the upstream reference site will guide selection of stressors for laboratory studies.

A literature review revealed a large number of environmental contaminants produced axial skeletal deformities in fish. Cadmium, lead, zinc, and probably arsenic, were neurotoxic (Holcomb *et al.*, 1976; Bengtsson, 1974), and fractured vertebrae through tetanic muscular contractions (Bengtsson *et al.* 1975). Davis *et al.* (1975) noted lead produced scoliosis, black tail, caudal atrophy, fin erosion, and hemorrhaging in the caudal region of fish. Bengtsson (1974) found 0.2 ppm zinc caused fractures and hemorrhaging, and that concentrations above 4.3 ppm were lethal. Bengtsson *et al.* (1975) found that 70% of the fractures resulting from cadmium intoxication were in vertebrae 2 through 7; likewise Bengtsson (1974) found that zinc caused 83% of the fractures in vertebrae 4 through 7. There was a striking similarity between the type and location of cadmium, zinc and organophosphate-induced fractures, (Bengtsson, 1974; Bengtsson *et al.* 1975; Bengtsson *et al.*, 1985; Hiraoka *et al.* 1984), and fractures induced by electrical shock (Bengtsson *et al.* 1975).

Organophosphate pesticides induced a very specific and localized fracture of the 2nd to the 13th vertebrae (Bauman *et al.* 1983; Hiraoka, 1983; McCann *et al.* 1972). This involved organophosphate

inhibition of cholinesterase. Excess accumulation of acetylcholine at neuromuscular junction resulted in excessive muscular contraction and fractures (Bauman *et al.* 1983). Hiraoka (1984) found the fractures induced by cadmium and organophosphates to be identical. Baumann *et al.* (1983) postulated a short-term exposure to organophosphate pesticides caused skeletal deformities in white crappies in Lake Decatur. They found application of organophosphate pesticides in the agricultural area surrounding the lake corresponded with the time white crappies spawn. In another study concerning agricultural runoff, Humar *et al.* (1983) found Malathion caused skeletal deformities, broken skulls and darkening of the body. McCann *et al.* (1972) tested the six commercial organophosphate pesticides on bluegills. Hemorrhaging occurred as a result of fractures at the base of the neural or haemal arch of the centrum. The hemorrhages reabsorbed in 2 weeks, but the caudal region remained bent at a 20° angle. The fish swam normally and remained alert for several months.

Reports of axial skeletal deformities due to low pH are not common in literature. White sucker embryos exposed to pH 4.5-5.0 were severely deformed and died within 24 hours of hatching (Frojnár, 1977). Adult suckers exposed to pH 4.2 for four weeks developed muscular and spinal deformities and died several weeks later (Beamish, 1977). Deformities could have been from decalcification resulting from acidosis. Kennedy (1980) reported 76% mortality in lake trout (*Salvelinus namaycush*) embryos that were kept at pH 5.8 for 30 days. The remaining embryos were severely deformed with the entire caudal region detaching. By day 59, only 6% of the fry in this study were still alive, and they were small and edematous with short thick trunks and disproportionately large heads.

Organic waste released from municipal sewage treatment plants commonly reduces dissolved oxygen in surface waters (Walker, 1997; Stewart, 1967). Dissolved oxygen may undergo large diurnal fluctuations; photosynthesis will release oxygen in the day, and plant and animal respiration will deplete oxygen levels at night (Stewart, 1967). Low levels of dissolved oxygen reduce growth and feeding (Stewart, 1967; Casselman, 1978). At 2.5 ppm to 4.5 ppm dissolved oxygen and between 2.5°C to 7.5°C, Garside (1959) noted abnormalities of the head and trunk in lake trout embryos. When exposed to less

than 0.3 ppm dissolved oxygen, 39 out of 40 Pacific salmon eggs hatched alevin with severe posterior truncation (Alderdice, 1957).

Elevated temperature influenced vertebral development and produced axial skeletal deformities in fish (Gabriel, 1944; Kwan, 1974; Lindsey *et al.*, 1971). Number of vertebrae increased at lower temperatures, and decreased at higher temperatures. Increased rate of development at higher temperatures perhaps contributed to increased frequency of axial skeletal deformities (Kwan, 1974). Incidence of fused vertebrae increased from 0.05% at 7°C to 16.2% at 15°C. In a study of vertebral variations induced by temperature in cyprinodontid fish, Lindsey (1971) found the highest temperature, 31.2°C, to have 100% skeletal deformities, and the lowest temperature, 19.5°C, to have 65% skeletal deformities. At three intermediate temperatures skeletal deformities ranged from 25% to 32%.

Tryptophan is an essential amino acid for fish. Tryptophan-deficient diets of fish increased incidence of cataracts and scoliosis or lordosis. (Akiyama *et al.*, 1986; Walton *et al.*, 1983). Ascorbic acid is an antioxidant, co-factor in collagen synthesis, a regulator of steroid synthesis, a growth activator in wound healing, a modulator of the hexose monophosphate shunt, and an inactivator of hepatic microsomal hydroxylases (Hardie, 1991). The ascorbic acid requirement for fish ranges from 25-50 mg/kg body weight/day (Lovell *et al.*, 1991). Ascorbic acid deficiency produces vertebral lesions including scoliosis, lordosis and focal hemorrhage.

Contaminant-induced vitamin C depletion and disrupted collagen metabolism is one potential basis of fish skeletal deformities (Bengtssen. 1979; Mauck *et al.* 1978; Mehrle *et al.* 1975, 1982). Arochlor 1254 (a PCB mixture) increased calcium levels and decreased collagen, hydroxyproline, and vitamin C in bone (Mauck *et al.* 1978). Arochlor 1254 and toxaphene may cause backbones to be more susceptible to fracture during times of stress (Mauck *et al.* 1978; Mehrle *et al.* 1975). Mehrle *et al.* (1982) found that fish taken from the Hudson River, which has high levels of PCB congeners and lead, had significantly decreased elastic limits (point at which permanent damage occurs), decreased rupture point (force causing bone failure), decreased stiffness (modulus of elasticity), and decreased toughness (energy

absorbed at bone failure). Degraded mechanical properties of bone were associated with inhibition of growth and development of fish exposed to organic contaminants (Mehrlé *et al.* 1982).

Skeletal deformities resulted from exposure to the organochlorine pesticide kepone and bleach kraft mill effluent (BKME) containing tetrachloro – 1,2 – benzoquinone (TCQ) (Bengtsson *et al.* 1987; Hansen *et al.* 1977). Bengtsson *et al.* (1987) found that 0.5 µg/L of TCQ caused 76% deformities in the last five vertebrae. Likewise, in a field study BKME was found to cause up to 59% deformities in the last four vertebrae (Bengtsson, 1991). The deformities documented were scoliosis, fusion, dislocation, and asymmetric vertebrae (Bengtsson, 1991; Bengtsson *et al.* 1987). Kepone poisoning is characterized by scoliosis, darkening of the caudal region, hemorrhaging of the brain and body, edema, fin rot, uncoordinated swimming, and cessation of feeding (Hansen *et al.* 1977).

In an inbreeding experiment rainbow trout fry developed a lethal body curvature soon after the reabsorption of the yolk sac. The behavior of the fry suggested the curvature was of neuromuscular origin (Aulstad *et al.* 1971). Both Atlantic salmon and brown trout developed compressed and often fused vertebrae in the anterior region of the axial skeleton (McCay, 1986; Poynton, 1987), and the brown trout also had scoliosis, pleural ribs that were twisted at the distal ends, curved neural spines, shortened upper and lower jaws, and up to a 25% reduction in the number of vertebrae (Poynton, 1987). Zebrafish inbreeding-induced deformities were characterized by an upward inclination of the head, flared opercula, lordosis and scoliosis (Prion, 1977).

Fisk *et al.* (1998) found the transfer of hydrophobic organochlorines from mother to eggs in freshwater fish varied within and between species and with the hydrophobicity of the organochlorine. Progeny of sheepshead minnows exposed to 0.8 µg/L kepone had significantly reduced growth and some had scoliosis (Hansen, 1977). Davis (1984) found that 50% of the progeny of fish exposed to 1.0 ppm dibutyl-phthalate exhibited vertebral fusion, deformed centra, and irregular neural and haemal spines.

In addition to xenobiotics, several infectious agents cause skeletal deformities in fish. These include viruses, bacteria, and parasites. Regarding the former, infectious hematopoietic necrosis (IHN)

virus is a widespread infection in captive and wild salmonid fishes in the Pacific Northwest. The infection usually occurs in very young fish, causing acute mortalities due to destruction of the hematopoietic tissue. However, about 1-4 % of rainbow trout that have recovered from the acute form of the disease develop spinal deformities (LaPatra *et al.* 2001). Coldwater disease, caused by *Flavobacterium psychrophilium*, is a common bacterial disease of salmonids in the Pacific Northwest that also causes skeletal deformities and spinal deformities in a similar manner as IHN. Bacteria may reside in the central nervous system and meninges in coho salmon (*Oncorhynchus kisutch*) that have recovered from systemic infections and these fish exhibit both spiral swimming behavior and skeletal deformities such as lordosis and scoliosis (Conrad and DeCrew 1967; Kent *et al.* 1989). While this bacterial infection is most noted in salmonids, cyprinid fishes and suckers are also susceptible to the infection.

Several parasites have been associated with spinal deformities, the myxozoan, *Myxobolus cerebralis* is probably the most well known. This parasite causes whirling disease in salmonid fish due to infections of the cartilage and bone of the head and vertebrae. In addition, the disease is characterized by scoliosis and lordosis due to dysplastic bone in the vertebrae (Hedrick *et al.* 1998). Whereas *M. cerebralis* is host-specific to salmonids, several other *Myxobolus* spp. infect vertebrae and cause similar changes. Vertebral anomalies are associated with *Myxobolus sandrae* infections of yellow perch (*Perca fluviatilis*) in Scotland (Treasurer 1992; Lom *et al.* 1991). Other examples include *M. ellipsoides* in chub (*Leuciscus cephalus*) in England (Bucke and Andrews 1985), *M. cartilaginis* in centrarchid fishes in the United States, and *Triangula percae* (Myxozoa) in yellow perch in Australia (Langdon 1987). Microsporidia may also cause spinal deformities. We recently described a microsporidian, *Pseudoloma neurophilia*, from the zebrafish (*Danio rerio*) (cf. Matthews *et al.* 2001). This parasite infects the central nervous system, ventral nerve roots, and occasionally the vertebrae and surrounding muscle. Infected fish exhibit scoliosis and lordosis, particularly when affected by the latter two manifestations of the disease. Larval digenetic trematodes may induce severe cartilage proliferation (Blazer and Gratzek 1985), and thus helminth parasites should also be considered as an agent for these changes.

B. Objectives

High prevalence of skeletal deformities was reported in juvenile fish from the Newberg Pool of the Willamette River in the early 1990s (Ellis, 2000). Prevalence of skeletal deformities in juvenile northern pike minnow ranged from 2-5% near Corvallis and from 26-74% in Newberg Pool between 1992 and 1994. This was confirmed in the spring of 2000 by Markle *et al.* (2002) and this study distinguished caudal and axial skeletal deformities. Of 15 species of fish examined, an axial skeletal deformity rate higher than 25% was detected in 10 species. Deformity rates varied substantially between species. For example, axial deformity rates were 80, 48, and 25% for chiselmouth chub, northern pike minnow, and bluegill sunfish, respectively. The total number of axial skeletal deformities was significantly lower near Salem (Wheatland Ferry) than in Newberg Pool.

Despite almost a decade of monitoring juvenile fish at Newberg Pool, likely explanations for high prevalence of skeletal deformities are lacking. The general objective of this proposal is to increase ability to explain this phenomenon. The approach involves a combination of literature, museum, field, and laboratory research. One important premise of this proposal is that identifying a single cause for a problem in a natural population in the open environment is typically difficult and sometimes impossible. In many cases a weight of evidence approach is most reasonable. The following study design addresses this limitation. Accomplishing the objectives below will allow integration of information from the literature, observations on museum specimens, field measurements of environmental conditions and deformity rates, and controlled laboratory experiments. The outcome of this integration will greatly increase our ability to identify environmental stresses that contribute to prevalence of axial skeletal deformities in juvenile fish in the Willamette River.

Objective 1: Continue to investigate historical prevalence of skeletal deformities in juvenile fish from the Willamette and other large Pacific Northwest rivers through examining the literature, museum and new field collections. Examination of Oregon State University museum collections indicates an axial

skeletal deformity load (see Fig. 1 for definition) of 0.12 in Willamette River juvenile northern pike minnow for 1952 (Markle *et al.*, 2002). They report a load of 0.25 for the Deschutes River that same year. This provides valuable context for new field work. In order to relate new data on water chemistry and environmental contamination to the problem we will determinate axial skeletal deformity rates in juvenile chiselmouth chub and northern pike minnow from two locations in Newberg Pool and one upstream site in 2002 and 2003. Histopathological examination of deformed vertebrae will assess potential contributions of infectious agents to development of these lesions. Laboratory culture of vertebral tissue from field-collected fish for identification of bacterial and viral pathogens will augment histopathology.

Objective 2: Compare and contrast water physical and chemical conditions, and concentrations of persistent bioaccumulative toxicants (PBTs) in adult northern pike minnow ovaries from Newberg Pool and one upstream sites in 2002 and 2003. Sonde probe measurements will provide data on physical and general water chemistry conditions from field sites. Comparing these conditions (e.g. pH, dissolved oxygen, and temperature) between Newberg Pool and the reference site provides an initial assessment of their potential roles in fish deformities. In situ sampling for waterborne bioavailable contaminants will provide information on their relative amounts at these sites. Maternal transfer of PBTs to eggs and hence developing embryos is a major problem in the Great Lakes and can lead to substantial losses of early life stage fish. Residue analyses of adult fish from Newberg Pool and an upstream site can identify the potential for a PBT to contribute to skeletal deformity rates. Collection of water from habitat in which early life stages of fish develop for chemical fractionation and analysis provides a novel approach to assessing potential roles of dissolved or suspended toxicants.

Objective 3: Conduct laboratory experiments with zebrafish and fathead minnows to assess potency of conditions identified in Specific Aim 1 and 2 to produce skeletal deformities. The general approach is to test environmental factors that differ in Newberg Pool from the reference sites in laboratory-reared, early life stage fish. Initial testing will focus on measurements from Sonde probes that correlate with axial skeletal deformity rates in fish collected from the field. In situ sampling for analyses of bioavailable

contaminants will provide another approach for identifying specific chemical to be tested in bioassays. Testing of PBTs in ovaries from adult fish from field sites that correlates with skeletal deformity rate will follow. Comparing and contrasting chemical fractions of water and suspended sediment from Newberg Pool and the reference site in terms of potency to produce fish skeletal deformities in laboratory experiments will assess contributions of dissolved or suspended toxicants. If histopathology and/or culture methods suggest an infectious etiology, then in vivo transmissions studies will be conducted with these agents in the bioassay systems.

C. Approach and Methodology

Objective 1: Field research will focus primarily on three areas: two sites on opposite banks of Newberg Pool, in the vicinity of RM 45-50; and upstream of Corvallis (RM 135). Over the past decade the area around RM 45-50 has consistently had the highest rates of fish deformities (Ellis 2000, Markle *et al.*, 2002). Corvallis provides a reference sites with a low prevalence of axial skeletal deformities (Ellis, 2000). The distance between Corvallis and Newberg Pool reduces likely mixing of individual adult or juvenile fish between the sites.

Operational Objective 1.1 Describe spatial and temporal patterns of fish deformities in the Willamette and another Oregon drainage.

Rationale.

Spatial - Most of the skeletal deformities in Willamette River fishes cannot be seen by superficial examination. However, x-ray analysis demonstrates high prevalence of axial skeletal deformities in Newberg Pool relative to other sites in the Willamette drainage Markle *et al.*, 2002. Preliminary data indicate that caudal skeletal deformities are high in other drainages but that axial skeletal deformities are not. Caudal deformities may be indicative of low-level stress while axial skeletal deformities, as seen in Newberg Pool, may indicate severe stress. If significant rates of axial skeletal deformities occur in fish from another Oregon drainage, this is a potential means of validation for observations from the

Willamette. Potential causative stresses for axial skeletal deformities in the Willamette River can be measured in another drainage to assess the association between prevalence of axial skeletal deformities and the stress.

Temporal – Temporal patterns of deformities show that fish upstream of Newberg Pool have had relatively high caudal deformity rates since about 1952 but that axial skeletal deformities did not reach high levels until about 1982 (Markle *et al.* 2002). There are additional historical samples (about 1400 specimens) between about 1944 and 1999 in the cataloged Fish Collection at OSU and many more in the un-cataloged collections. In addition, holdings at the California Academy of Sciences, University of Michigan, University of Washington, and US National Museum of Natural History contain relevant historical collections. As with spatial patterns, we anticipate that temporal patterns can help define the time period when stresses are present that may cause caudal and axial skeletal deformities. Again, the timing of deformity patterns can be used to corroborate cause and effect, if for example, a stress occurs at sites with high axial skeletal deformity rates, but not reference sites.

Task 1. Identify and radiograph historical collections of fishes from the following areas of the Willamette River – 1. Two sites within Newberg Pool and Corvallis.

Task 2. Identify and radiograph collections of fishes from an Oregon drainage outside of the Willamette, probably the Deschutes drainage.

Task 3. Calculate individual deformity loads.

Task 4. Calculate sample deformity frequencies.

Task 5. Analyze spatial and temporal patterns of axial skeleton and caudal deformity loads and frequencies.

Operational Objective 1.2. Describe seasonal changes in axial skeleton and caudal deformities in cyprinids in Newberg Pool and Corvallis.

Rationale

Because deformities occur early in larval or juvenile development, potential mechanisms of action include: maternal transfer of a contaminant through yolk, a waterborne environmental stress that is

especially prevalent in Newberg Pool, or a high frequency mutation that leads to vertebral malformations. The last possibility seems unlikely since 15 of 20 fish species exhibit high prevalence of axial skeletal deformities in Newberg Pool. Deformities associated with growth of elements rather than formation of elements could indicate chronic stress. Seasonal changes will indicate if there is differential mortality (Fig. 2) associated with deformities and if there are indications of chronic stress leading to progressive lesions in skeletal elements. We would expect that axial skeletal and caudal deformities in the “control area” and caudal deformities in Newberg Pool would stay constant over the year but that the more significant axial skeletal deformities in Newberg Pool would decline seasonally, as observed in chiselmouth in 2000 (Fig. 2).

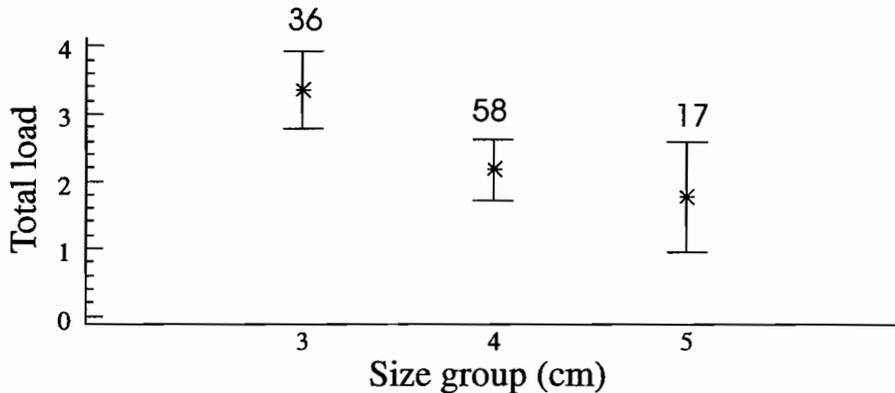


Figure 2. Relationship between total deformity load (sum of caudal and axial skeletal deformities) and size group (cm) in chiselmouth (*Acrocheilus alutaceus*) from the Willamette River, 2000. Stars indicate means; bars show 95% Bonferroni confidence interval; numbers indicate sample size.

Task 1. Select a reference site that has a similar native cyprinid fauna, upstream of Newberg Pool, and has axial skeletal deformity loads and frequencies significantly lower than Newberg Pool fishes. Corvallis meets these criteria.

Task 2. Collect samples of larval and juvenile cyprinids over the season from about June through October in Newberg Pool and the reference site.

Task 3. Radiograph specimens with ossified fins and vertebrae to determine deformities.

Task 4. Prepare trypsin-cleared and alizarin/alcian blue-stained preparations of larvae and unossified juveniles to determine developmental stage, size and age at which deformities occur.

Task 5. Examine a subset of lapilli otoliths of deformed and normal fish of comparable sizes to estimate ages in days.

Axial skeleton deformities will be evaluated using radiographs and methods in Markle *et al* (2002). Vertebrae will be categorized as caudal - those of preural centrum 2 and all posterior, and those anterior of preural centrum 2 (axial skeletal). Vertebral deformities will be scored for multiple neural spines, multiple haemal spines, fused centra, and other fused elements (neural, haemal, erpural and hypural). Deformities in the unpaired fins (dorsal, caudal and anal) will be scored for fused or aberrant pterygiophores and fused or aberrant fin rays.

The analyses will be of deformity loads (the number of deformity categories per individual fish) and frequency of deformed fish in a sample. Both statistics will be calculated separately for axial skeletal and caudal categories since previous work has shown that caudal deformities can mask patterns of the more severe axial skeleton deformities (Markle *et al.* 2002).

Operational Objective 1.3 Investigate the role of infectious agents in axial skeletal deformities.

As parasites, bacteria and viruses can cause deformities in fishes, we will conduct routine diagnostic procedures and transmission studies to evaluate the presence and association of infectious agents in the lesions. The presence of parasites in lesions (both protozoan and metazoans) will be evaluated by careful histological examination. Bacterial cultures from the lesions and viral cultures from whole fish will be evaluated, the former concentrating on the presence of *Flexibacter-Flavobacterium* spp. as these are well-documented causes of these lesions. An infectious etiology will also be evaluated in an in vivo laboratory study by injection of lesion tissue into fish as described in Objective 3.

The visceral organs and lesions from affected fishes will be assayed for the presence of viruses using cell culture. We will use standard techniques routinely used in fish virology laboratories (Ganzhorn and LaPatra 1984). Suspect tissues will be assayed at dilutions of 1:10 and 1:100 using a variety of

available cell lines, concentrating on those from cyprinid fishes. This include FHM (fathead minnow), EPC (carp epithelioma), ZR4 (from zebrafish), and BF-2 (from blue-gill fin). Cultures will be incubated at 2 separate temperatures; 17 and 23 C, and all negative cultures will be blind-passed after 2 wk for an additional 2 wk culture.

If cultures exhibit cytopathic effects suggestive of viruses, the presence and identification of these agents will be further determined using standard techniques such as electron microscopy, or serum neutralizations (Ganzhorn and LaPatra 1994). Viral cultures would also be retained for future *in vivo* evaluations for their pathogenicity and ability to cause axial skeletal deformities.

Aseptic inocula will be obtained from affected vertebrae and spinal cords and cultured on a variety of media, including routine media for fish bacteriology (i.e., TSA and blood agar), and specific media of *Flexibacter-Flavobacterium* (e.g., Shieh and modified cytophaga agar) (see Holt 1994). Bacteria will be identified using standard morphologic and biochemical methods.

Affected and apparently normal fish of representative species will be examined by light microscopy for the presence of histological lesions. 30 fish of each species, and 10 controls (i.e., from low prevalence sites) will be euthanized by an overdose of MS-222, preserved in Dietrich's fixative, and processed for routine light histology. Most fish are small enough to be processed whole, and these fish will be examined by step-wise mid-sagittal sections. This allows for examination of almost all organs, including the vertebral column and brain. We will also examine visceral organs, especially the liver and kidney, for toxicopathic changes. Slides will be examined by both Drs. Jan Spitsbergen (i.e., morphological descriptions) and Michael Kent (pathogen identifications).

Objective 2.

Operational Objective 2.1: We will compare and contrast water chemical-physical conditions at two locations in Newberg Pool and the reference site (Corvallis). During spawning/early fish development (mid-April though mid-July in 2002 and 2003) we will deploy Sonde probes to *continuously* collect a set of physical/chemical data. This will include episodic events (stormwater) and other pulses of

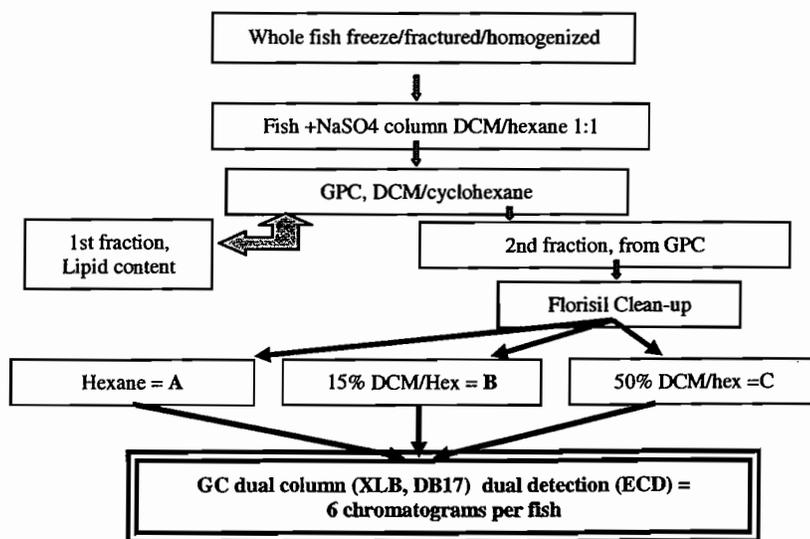
contaminants in the sampling scheme. We will continuously monitor (24 hours a day and 7 days a week) depth, pH, temperature, dissolved oxygen, ammonia, nitrate, total dissolved sediment, conductivity, and oxidation reduction potential. The YSI® field Sonde probe collects data for these parameters.

Chemical/physical parameters (ammonia, nitrate, oxidation reduction potential, pH, dissolved oxygen, total dissolved solids, conductivity, temperature) will be collected hourly via the deployed Sondes. The YSI® field Sonde probes will be deployed continuously from mid-April through mid-July (during spawning and early life development). We will also collect data within the river at multiple depths simultaneously from bottom to surface; in this fashion, we can develop a three dimensional picture of the variation of these parameters. Initially, our continuous Sonde deployments will be 1 ft from the river bottom. Depending on results from this study (Year 1 and information from the YSI® field probe data) we may change or expand the depth(s) sampled. One set of standard grab samples will be collected in May, and analyzed for organic contaminants and metals via standard US EPA protocols, (US EPA Method 508.1 and 200.8 or 200.9)

Operational Objective 2.2:

Maternal transfer of persistent bioaccumulative toxicants (PBTs) to eggs and hence developing embryos is a major problem in the Great Lakes and can lead to substantial losses of early life stage fish. We will collect ovaries from and northern pike minnow at Newberg Pool and the

reference site. We will chemically analyze the ovaries for a select group of PBTs. Analysis will be by GPC (gel permeation chromatography) and GC-ECD as previously described (Sethajintanin *et al.*, 2001) see schematic. The analyte list will be the same as described below. Chlorinated dibenzo dioxins and chlorinated dibenzo furans produce toxicities at extremely low concentrations. Axys Analytical



(Vancouver, BC) offers high sensitivity with excellent quality control. They will subcontract these analyses. We will collect 5 individual fish at each site, a total of 10 samples will be analyzed each year.

Operational Objective 2.3: Bioavailability of pollutants is the accessibility of a chemical for biological assimilation and possible toxicity. Federal/state regulatory agencies typically rely on analytical methods that entail vigorous extraction of matrices with organic solvents (pesticides) and strong acids (metals) that may in fact not accurately estimate the magnitude of environmental risk. The relevancy of such methods to toxicity is often not considered, thus decisions are based on data that is often not relevant for prediction of potential exposures and risk. There is compelling evidence that the chemical quantities recovered by vigorous extraction/digestion fail to predict bioavailability (Alexander, 2000). We will deploy passive sampling devices (PSD) for passive in-situ monitoring of bioavailable organic contaminants and diffusion gradient thin films (DGT) for inorganic bioavailable metals. The PSD/DGT devices are placed in cages with Sondes and submersed continuously from mid-April to mid-July. Every three weeks the PSDs and DGTs are collected for analysis and replaced with new devices. In this way episodic events and/or other pulses of contaminants will be sampled. Our hypothesis is that bioavailable contaminant levels better represent chemical exposures in ecosystems than vigorous extraction of water. The PSD/DGT method also provides continuous sampling. Episodic events strongly influence temporal levels of bioavailable contaminants effecting early life stages development of fish.

Bioavailable contaminant levels and transformations must be understood before their potential contributions to axial skeletal deformity rates can be discerned. Understanding bioavailable contaminant levels (Anderson and Johnson, 2001) in a complex ecosystem, such as a contaminated site, is an important part of identifying chemical stressors (Alexander, 2000). In addition, understanding the variability (spatial and temporal) of bioavailable contaminants in the context of “natural” cyclic processes, such as seasonal variation in rainfall is critical. Knowledge of bioavailable load (van Leeuwen, and Pinheiro, 2001) and fluctuations is necessary to assess potential for bioaccumulation (Hofelt and Shea, 1997; Axelman, Nav and Broman, 1999) and biomagnification (Herve *et al.*, 1995; Herve, *et al.*, 1995; Moring and Rose, 1997) to produce toxicity, such as axial skeletal deformity.

Health risk and fate depend on a “contaminants” chemical form. For organic and inorganic compounds, aquatic toxicity data, water quality criteria, and threshold limit values are based on dissolved concentrations and not total (EPA 822-Z-99-001, 1999) residue levels (Campbell, 1995; Nowell and Resek, 1994). Determination of the bioavailable fraction is therefore important. Presented below one can (Nowell and Resek, 1994) see how different conceptual approaches can lead to significantly different estimates of exposure.

↑ Estimated Exposure	Compound Release	Analytical Chemistry	Biological Response
	LOADING	Non-recoverable	Not Environmentally Available
		Analytically Recoverable	Not Environmentally Bioavailable
			Not Pharmacologically Bioavailable
		BIOLOGICALLY RECOVERABLE	

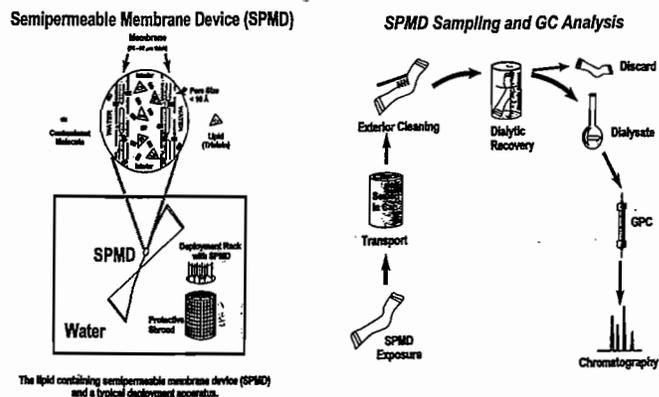
A sampling scheme that incorporates episodic events is necessary for understanding contaminant effects in ecosystems. Episodic events like storms (Leecaster, Schiff, and Tiefenthaler, 2001; Buren, Watt, Marsalek, 1997; Makepeace, Smith, Stanley, 1995) can greatly contribute to introduction of contaminants in ecosystems. Stormwater runoff (Schiff and Tiefenthaler, 2001) and urban overflows (Tran, Young and Zeng, 1999) are widely believed to be one of the largest sources of contaminants to some surface waters. In addition, to the direct input of pollutants during runoff, there is also concern that severe sediment disruption may re-mobilize bioavailable contaminants (Petty *et al.*, 1998). Organic chemical contaminants increase in rivers during and immediately following flooding events. Flooding ‘stirs’ sediments possible re-mobilizing contaminants (Petty, *et al.*, 1998) however, not just particulate bound contaminants but bioavailable contaminants as well. The combined detection of organic and metal contaminants will be of considerable usefulness to a comprehensive evaluation of episodic events.

In-situ passive sampling with PSD/DGT devices employs extractions and analysis that are simple and straight forward. The equipment used is readily available; for example, GC-ECD (gas chromatography electron capture detection), HPLC (high performance liquid chromatograph) and ICPAES (inductively coupled plasma atomic emission spectroscopy) or anodic stripping voltametry (ASV). With this approach, bioavailable organic compounds and bioavailable elements can be determined routinely and data will be more toxicologically relevant. Most importantly the PSD/DGT devices allow for time-averaging of contaminants, so pulses of contaminant releases are sampled.

PSD were selected since they sample only organic chemicals (lipophilic) in solution (dissolved) (Petty, *et al.*, 1995; Huckins, *et al.*, 1999) while excluding those sorbed on organic matter or particles. Studies of PSD bioavailable organic compounds have been correlated directly with bivalves (Hofelt and Shea, 1997; Axelman, Naes, Broman, 1999; Herve *et al.*, 1995; Prest *et al.*, 1992; 1995; Herve, *et al.*, 1991; Moring and Rose, 1997), catfish (Gale *et al.*, 1997), and goldfish (Wang *et al.*, 1998; Wang *et al.*, 1999). Furthermore, PSD (and DGT) sampling integrates exposure over the entire sampling period to approximate actual exposure. Figure 2.1 (Huckins *et al.*, 1999) illustrates the construction, deployment configuration, extraction and analysis scheme when analysis is by GC-ECD. Studies detailing the development and application of PSD for passive in-situ monitoring of aquatic bioavailable organic contaminants has been previously presented (Huckins *et al.*, 1999; Prest *et al.*, 1992). PSD have been used for over a decade and their effectiveness for determining bioavailable organic compounds is established. The PSD are used for contamination monitoring and toxicity assessment.

Apart from the development of equilibrium techniques to detect trace metal species (Anderson, 1999), in-situ methods (Anderson and Markowski, 2000) avoid equilibrium problems and emphasis on in-situ types of techniques (Buffle *et al.*, 1997) is likely to increase (Zhang and Davison, 2001). DGT will

Figure 2.1: PSD construction, deployment, extraction & analysis scheme



be used for quantitative determination of bioavailable (labile-dissolved) metals in-situ. The theory and sampling regime for DGT probes have been previously reported (Zhang and Davison, 1994; Zhang and Davison, 1995). The simple procedure uses a layer of Chelex resin impregnated in a hydrogel to bind the metals. A diffusive layer of hydrogel and filter overlies the resin-layer.

Ions have to diffuse through the filter and diffusive layer to reach the resin layer.

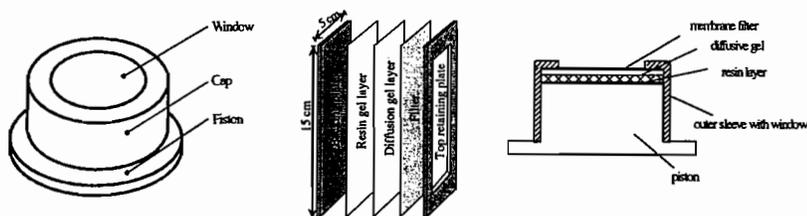
It is the establishment of a constant concentration gradient in the diffusive layer that forms the basis for measuring metal concentrations in solution quantitatively without the need for separate

calibration. Exhaustive testing of DGT has been performed with this gel composition and measurements establish that the diffusion coefficients for simple ions in this gel are indistinguishable from published values in water (Zhang and Davison, 1994; Zhang and Davison, 1995). The DGT device is deployed for a known time and then the mass of metal on the resin is measured after elution with acid by; for example, AAS (atomic absorption spectrometry), ICP-AES (ICP atomic emission spectrometry), ICP-MS or ASV (anodic stripping voltametry). Providing the temperature is known, the concentration in solution can be calculated.

When deployed in water DGT measures labile species (inorganic and organic complexed metals). Above a low threshold value, the measurement is independent of solution flow. DGT has been deployed in-situ in rivers, lakes, estuaries and the deep sea (Zhang and Davison, 1994; Zhang and Davison, 1995). Its built-in pre-concentration gives it excellent sensitivity (10^{-12} moles/L) and avoids contamination problems, see Figure 2.2 (Zhang and Davison, 1994). Parallel deployment of two DGT units of different diffusive layer thickness allows accurate measurement under even low flow conditions.

Measurements of the total amount of a compound in a particular sample matrix actually reveals very little, about its possible mobility, toxicity or biogeochemical function. We will apply parallel

Figure 2: DGT construction



approaches; the first approach will utilize in-situ monitoring probes that by design measure bioavailable chemical species. The second approach is based on classical methods.

Water samples will be collected by deploying PSD and DGT probes in protective mesh cages at designated sites. Individual PSD tubes (1 to 5) and two DGT will be included on each buoy arrangement. Each PSD/DGT cage is suspended with “marker buoy-rope-float-cage-cable-anchor” arrangement. The buoy arrangement ensures the cages stay suspended at the correct depth and the cages stay in the deployed locations. The cages can be deployed at any depth from sediment surface to the water surface. Initially, our cage deployments will all be at ca 1 ft from the river bottom.

Each individual deployment will be for approximately 3 weeks, and we will continuously deploy cages from mid-April through mid-July. Deployment times need to be long enough to ensure PSD concentrations are above detection limits (DL), we have performed basic calculations necessary to insure 1-5 PSD tubes will provide levels 10-50 times DL, based on existing information. We will establish local deployments times, number of PSD tubes to composite, and extraction efficiencies to ensure detection of as many analytes as possible from our list. The Newberg Pool site will be duplicated, we will be collecting simultaneously at 3 sites. Initial data from our other studies indicates that variance between replicate bioavailable contaminants in samples is relatively small; therefore, the necessary replication at each site to provide the statistical power required to differential sites is modest. Sampling the first year will ensure we have sufficient statistical power to make direct comparisons (see data analysis below).

Although PSD and DGT techniques are capable of isolating many compounds, we will *at a minimum* measure the following analytes during our study: organochlorine compounds (e.g. DDT, DDD, DDE, dieldrin, and ca 20 select PCB congeners), organophosphate/other pesticides (e.g. chlorpyrifos), polynuclear aromatic hydrocarbons (e.g. benzo(a)pyrene) and metals (e.g. Fe, Cd, Cu, Zn). All pesticide samples will be collected in 1L amber jars and metals samples will be transport (DGT) or collected in HDPE bottles. All pesticide samples will be kept on ice during transport and stored at <-20C until analysis, metal samples at 40C. Pesticide will be extracted within 7 days of collection.

Data quality objectives will include: relative percent difference of field duplicates at $\leq 25\%$ (laboratory duplicates $< 20\%$), recoveries of fortified samples at 70-130%, spiked samples and SRM from NIST or BCR (Community of Bureau of Reference of the European Communities) (Quevauviller *et al.*, 1993) (both are developing reference materials for speciation studies) will be analyzed, as well as, QC objectives as listed below.

Quality Assurance and Quality Control: As a GLPS (good laboratory practices standards) program, we have a standard operating procedures manual and a quality assurance plan in place, both have withstood several audits. Sampling protocols have been established as part of our quality assurance plan. All samples will be kept in coolers, at 4C, during transport. An example of some of our QC requirements includes: 10% field deployment duplicates, 4-5 point calibration, linear calibration correlation coefficient > 0.99 , initial fortification recovery studies recovery of 70-130%, continuing calibration samples 10% of total batch, continuing analysis of blanks 10% of total batch, formal method detection limit according to EPA standard procedures (40 CFR 136), analysis of real world samples precision estimates (25% RPD).

Data Analysis & Statistics:

A statistical treatment (including: F-test, Tukey-Kramer, and first order analysis of variance) of the results will be obtained, which will yield estimates of accuracy, bias, precision and will allow determination of significance of difference between episodic events, seasonal variability, and depth dependence on bioavailable contaminants. We will also investigate associations between macro chemical physical characterization and changes in bioavailable contaminants.

Objective 3: To further investigate the cause(s) of skeletal deformities, an early life-stage fish bioassay will be developed to test both chemical and infectious agents. In recent years the United States Geological Survey and others have monitored the Willamette River for priority pollutants, including metals and persistent organic pollutants (PCBs, DDT, other organochlorine pesticides, chlorinated dioxins and

furans), as well as many less persistent pesticides. However, the monitored chemicals represent only a fraction of the potential load to the Willamette River. There are over 84,000 regulated chemical substances in the USA, and approximately 1000-1500 new chemicals are manufactured each year. Worldwide, the total synthetic and naturally-occurring xenobiotics probably number in many hundreds of thousands; this is undoubtedly a conservative estimate given that Chemical Abstracts Service has registered over 15 million organic and inorganic substances (as of 2001), and over 1,800,000 of these are readily commercially available (<http://www.cas.org/cgi-bin/regreport.pl>). Of particular interest are the approximately 150 pesticides, totaling 4 million pounds applied annually to crops grown in the Willamette Valley. This does not include other important pesticide uses such as urban use, rights-of-way, rangeland, and forestry. In addition to manufacturing and chemical use in agriculture, other sources of chemicals in the Willamette include municipal sewage treatment works (STWs) effluents, septic systems, leach fields, hospital waste (both pre-treated and untreated sewage and solid wastes), untreated STW sewage from excessive precipitation (when storm drains are tied to domestic waste conveyance systems) and STW failures, landfill runoff and leachate, and confined animal feeding operations.

In considering the exposure universe of all chemicals, many have the potential to reach surface water, but only a small fraction is monitored. Major determinants for chemical monitoring programs are the availability of specific and sensitive analytical methods that meet quality assurance standards and cost. A large majority of pollutants are not monitored because appropriately specific and sensitive and/or cost-effective analytical procedures do not exist. This is particularly true for what are called “emerging pollutants,” such as endocrine disrupting compounds, pharmaceuticals, and personal care products (Daughton and Terres, 1999). Also of concern is the fact that once released, all chemicals undergo transformation in the environment through biotic, chemical, and photochemical processes. Yet little is known about the fate or toxicity of most transformation products, and methods for monitoring and analysis are even more elusive than for the parent compounds.

Operational Objective 3.1: Chemical fractionation of Willamette River water. Clearly, to further investigate the cause(s) of fish deformities in the Willamette, it is not feasible or practical to

perform a bioassay on all suspected pollutants and their transformation products, singly or as mixtures. Instead, we will separate pollutants from water based on their physical and chemical properties. For example, all chemicals can be broadly classified as organic and inorganic. The organics (carbon based) can be further segregated based on such key physical-chemical properties as volatility (volatile, semi-volatile, non-volatile), water solubility (polar, non-polar), or ionization state (neutral, acidic, basic). An organic chemical's functional groups determine its classification. We propose to initially bioassay chemical classes using a directed fractionation and characterization strategy. We will use knowledge of chemical functionality, as well as published physical and chemical properties, as a guide in the development of fractionation methods. The results of these bioassays should allow us to efficiently focus our efforts by identifying those fractions that are most toxic, as well as "ruling out" certain classes of compounds.

Lewtas (1988) characterized and identified of mutagens and carcinogens in the complex mixture associated with combustion sources using a bioassay-directed fractionation procedure. Isolated fractions were tested using the Ames mutagenesis assay. The fractionation strategy employed traditional solvent extraction techniques commonly used in the analysis of environmental samples. We plan to employ solid phase extraction (SPE) techniques to fractionate Willamette River water samples prior to bioassay. A common solid phase is C18 (octadecyl silane), which is known to selectively retain semi-volatile, non-polar neutral organics. Large, very non-polar analytes, although well retained on C18 sorbents, can be difficult to elute as the non-polar interactions between analyte and sorbent are very strong. If a less retentive phase (such as C8, C6, C4, C2) is used, the analytes will still be retained, but they can be eluted more easily. Semi-volatile basic compounds can be selectively retained on C18, C8, C6, C4, or C2 sorbents in which the silane backbone is not encapped, resulting in exposed silanol (SiO^-) groups. Basic compounds are retained by both non-polar and ionic retention mechanisms. An alternative for the retention of basic compounds is a solid phase consisting of strong cation exchange (SO_3^-) and C8 mixed mode chemistries. Additional cation exchange sorbents include benzenesulphonic acid, ethylbenzene sulphonic acid, and propylsulphonic acid, which are classified as strong cation exchangers. They maintain

a permanent positive charge over the whole pH range (pH 1-14). A carboxy propyl phase is a weak cation exchanger with a pKa of 4.8. It is used for the extraction of cations that exhibit a positive charge at pH 6.8 or higher. Semi-volatile acidic compounds can be selectively retained with anion exchange phases such as a primary/secondary amine phase; a weak anion exchanger with pKa's of approximately 10.1 and 10.9. The aminopropyl phase is a weak anion exchanger, with a pKa of 9.8. The quaternary amine phase is a strong anion exchanger with chloride as the counter ion. It maintains a permanent positive charge over the whole pH range (pH 1-14). In general, when using cation or anion exchangers, the pH can be adjusted so that acidic or basic compounds can be selectively retained based on their pKa value. As an alternative to silica based sorbents, highly crosslinked resin-based sorbents (hydroxylated polystyrene-divinylbenzene, polystyrene-divinylbenzene) are capable of retaining analytes of a wide range of polarities. The very accessible high surface area of this nonpolar sorbent retains very polar and water soluble analytes. Table 3.1 gives a comprehensive listing of available solid phases that selectively remove organic chemical classes from a complex mixture in an aqueous sample, based on their physical and chemical properties.

Table 3.1. Solid Phase Sorbant Phases

Resin based sorbents: primary interactions are strongly NON-POLAR

Hydroxylated polystyrene-divinylbenzene
Polystyrene-divinylbenzene

Silica based non-polar sorbents: primary interactions are NON-POLAR

Endcapped and Non-endcapped. Non-endcapped sorbents exert stronger polar and ionic secondary interactions than endcapped sorbents

C18 Octadecyl
C18 Octadecyl monofunctional
C8 Octyl
C6 Hexyl
C4 Butyl
C2 Ethyl
CH Cyclohexyl
PH Phenyl
CN Cyanopropyl

Silica based mixed-mode sorbents: primary interactions are NON-POLAR and IONIC

HAX C8 and strong anion exchange

HCX C8 and strong cation exchange
 HCX-3 C18 and strong cation exchange
 HCX-5 C4 and strong cation exchange
 Multimode C18, strong anion and cation exchange

Silica based ion exchange sorbents: primary interactions are IONIC

Silica based anion exchangers	Secondary interaction (aqueous matrix)
NH ₂ Aminopropyl	Weak non-polar
PSA Primary secondary amine	Weak non-polar
SAX Quaternary amine	Weak non-polar
PE-AX Quaternary amine	Weak non-polar
Silica based cation exchangers	
CBA Propylcarboxylic acid	Weak non-polar
SCX Benzenesulphonic acid	Medium non-polar
SCX-2 (PRS) Propylsulphonic acid	Weak non-polar
SCX-3 Ethylbenzene sulphonic acid	Strong non-polar

Bioassay-directed fractionation may identify chemical classes in Willamette River water that cause axial skeletal deformities. Prior to bioassay with early life stages of zebrafish, Willamette River water will be fractionated with the solid phase types listed above. Fractionation methods may employ these solid phases singly or in series. Water samples from two sites in the Newberg Pool and Corvallis will be extracted. The extracted water, containing all elements of the complex mixture of potential toxicants except those selectively excluded, will be used for the bioassay. For example, organic acids, organic bases, and neutral organic compounds can be selectively removed and each fraction bioassayed. If the incidence of axial skeletal deformities is reduced in the fraction with acidic compounds removed, then this fraction will be studied further. By varying the pH of the water and the strength of the solid phase anion exchange, bioassay results should narrow the chemistries of interest. To validate the method we will spike water with various mixtures of chemicals representative of priority pollutants, persistent organic pollutants, pesticides, endocrine disrupting compounds, pharmaceuticals, and personal care products.

Willamette River water with or without solid phase extraction (approximately 1 liter) will be reduced to 100 milliliters by rotary evaporation and then freeze dried. Freeze drying the concentrated sample rather than taking the sample to dryness in the rotary evaporator will minimize the loss of semi-volatile compounds. Freeze drying will remove small amounts of solvents required in the solid phase extraction, especially methanol which forms an azeotrope with water. Freeze dried samples will be reconstituted in the standard water for bioassay. The potency of individual fractions will be investigated by varying their concentration in the standard water. A clear dose response will be further evidence of toxic effect.

The goal of this bioassay-directed fractionation and characterization strategy is to further refine our search for the cause(s) of the high prevalence of fish deformities in Newberg Pool. In addition to focusing our effort, this approach has the potential to “rule out” entire classes of chemicals as causative agents. Once the field of toxic agents has been narrowed, it may be feasible to further characterize those groups of extractants identified by the bioassay. For example, for a fraction that produces low incidence of axial skeletal deformities, the extractants remaining on the solid phase sorbent can be eluted and analyzed by gas or liquid chromatography with mass selective detection.

Operational Objective 3.2: Bioassay of selected environmental stresses in early life-stage zebrafish and fathead minnows. Comparing and contrasting environmental stresses at Willamette River locations that differ in axial skeletal deformity rates can identify potential contributors to the problem. Controlled laboratory experiments can test whether specific stresses produce axial skeletal deformities under conditions relevant to the field sites. This can substantially strengthen the weight of evidence approach we propose herein. High prevalence of axial skeletal deformities occurs in many fish species in Newberg Pool (Markle *et al.*, 2002). Elevation of deformity rates in 10 of 15 fish species strongly argues against a heritable genetic basis for the problem. It also indicates use of a particular fish species that occurs in Newberg Pool is not necessary for experimental work. This is especially important since laboratory spawning methods for northern pike minnow and chiselmouth chub are not readily available. Use of fish species broadly-used in experimental work that readily reproduce in the laboratory offers two

major advantages: (1) Extensive literature on developmental and general biology is available for design and interpretation of experimental work. (2) Methods for culture and availability of stock are established. This greatly reduces time and expense requirements for starting laboratory work. The zebrafish is emerging as an experimental animal of choice in developmental biology (Fishman, 2001). There is an existing colony at our Marine and Freshwater Biomedical Center core facility, where space is available for the work we detail below. To avoid unexpected species-specific phenomenon, we will replicate key experiments in fathead minnows. This fish is a bioassay model the United States Environmental Protection Agency often prefers.

The axial skeleton of fish differentiates at early life-stages. Ossification of primordial cartilage masses into vertebrae progresses from cephalic-to-caudal (head-to-tail) in a time and temperature-dependent manner. For example, in zebrafish at 28.5°C the 3 most anterior vertebrae begin to form at 6-7 days post-fertilization and all vertebral centra are present after 9 days (Eisen, personal communication). Development of ribs, neural arches and haemal arches is complete 21 days post-fertilization.

We will test stresses we identify in Objectives 1 and 2 and fractions of Willamette River water from the three field sites for laboratory bioassays. Collection of *in situ* water quality data, bioavailable contaminants in water at each location, and water for fractionation, will occur during April-June. This corresponds with the time of early development for fish exhibiting high axial skeletal deformity rates. Collection of ovaries from adult fish will precede that normal spawning time (May). Oregon Department of Fish and Wildlife and Oregon Department of Environmental Quality will assist in sampling and selection of collection times (letters appended).

Exposures of early-life stage fish will correspond with developmental stages of the axial skeleton. Duplicate groups of 25 larval fish will receive 24 or 96 hour duration waterborne exposures that begin at the time of formation of the 3 most anterior vertebrae. In the case of fractions from Willamette River water or bioavailable contaminants (Objective 2.3), the highest concentration will correspond to 10-times that present in the field. Screening bioassays for river water fractionation work will include controls plus two reconstituted concentrations of each fraction. Exposures to fractions or other environmental stresses

screening or field work indicates are worthy of close attention will be to a geometric series of five chemical concentrations, including control. This will yield exposure-response relationships with field concentrations in the mid-range. For a PBT identified in analyses of ovarian tissue for maternal transfer, another approach is more appropriate. Adult female fish (n=4 per concentration) will receive a single PBT intraperitoneal injection one month prior to onset of ovarian development. We have shown single intraperitoneal injections of fish with PBTs produce biological responses comparable to longer term feeding exposures. (Foster and Curtis, 1999). Five concentrations, including squid oil vehicle control will follow a geometric progression. The highest concentration will approximate the ovarian PBT concentration for Newberg Pool fish. We expect a minimum 10-fold magnification from whole-body dose to ovarian tissue. Twenty-five fertilized eggs from each female will be collected for study.

A brief description of general laboratory conditions for bioassay work follows: Well water is degassed, filtered through sand and activated charcoal, and then adjusted to pH 7.2. Survival of control embryos to one month of age is about 70%. Developing embryos and larvae will be reared in glass Petri dishes at $27\pm 1^{\circ}\text{C}$ in groups of 25. After onset of feeding, we will first transfer each group of fish to 500 ml beakers, and later 4 liter aquaria. We will rear fish to a standard length of 1 cm (about 6 weeks post-fertilization), euthanize them in 200 mg MS-222 per liter, and fix them in formalin. We will quantitate skeletal deformity rates via x-ray as in Objective 1.2.

Some viruses and bacteria are very difficult to culture, and thus the presence of an infectious agent may be best demonstrated with transmission studies (Kent and Dawe 1993). We will collect vertebral lesions from 20 affected fish aseptically by disinfection of the surface of the fish and dissecting instruments. Tissues will be combined together, mixed with sterile saline, and distributed with a stomacher. Material will be filtered down to 15 μm , and fish will be injected with 0.2 ml/fish. Two species of fish will be used in the study. 30 fish/species will be injected with the lesion material, and 30 controls will be injected with sterile saline.

All fish will be maintained in separate flow-through aquaria at the Salmon Disease Laboratory. Fathead minnows will be held at 20 C., while zebrafish will be held at 25 C. Fish will be fed with a

commercial diet daily. All moribund and dead fish examined by histology. The study will be terminated after 9 mo. or at an earlier date if recipient fish develop the skeletal deformities.

We will calculate axial skeletal deformities rate for each treatment replicate as percent. After arcsine transformation of percent data, we will perform one-way analysis of variance to determine statistical significance of any treatment effect ($p < 0.05$).

**D. WORK PLAN AND TIMELINE FOR FIELD AND LABORATORY WORK ON FISH DEFORMITIES IN
THE WILLAMETTE RIVER FROM MARCH 2001 THROUGH JUNE 2004**

2002

	___ Environmental ___	___ Fish Residue Analyses ___	
Prepare For	Monitoring		Initial
Field Work	___	___ Fish X-Ray Analyses ___	Report
		___ Chemical Fractionation of Water ___	
	___ Fish Collection ___	___ Laboratory Fish Bioassays and Histopathology ___	

2003

Prepare For	___ Environmental ___	___ Fish Residue Analyses ___
Field Work	___	Monitoring
		___ Fish X-Ray Analyses ___

| _____ Bioassays _____ |

| _____ Chemical Fractionation of Water _____ |

| _____ Fish Collection _____ |

| _____ Laboratory Fish Bioassays and Histopathology _____ |

2004

| _____ Bioassays _____ | Final Data Analysis _____ |

And Report

Preparation



E. Project Management, Collaboration and Matching Funds

All of the co-investigators will meet on a formal basis every two months to provide updates on research progress. Smaller group meetings will occur to coordinate field work at least twice each month. Near the end of the first and second years of the project, scientists and graduate students will present research results at regional and national meetings. Prior to these meetings, all project staff will meet to review presentations.

The budget commits \$243,758 in cost share. This demonstrates a strong institutional commitment to this project.

Planning this proposal included establishing formal collaborations with two key state agencies: The Oregon Department of Fish and Wildlife, and Oregon Department of Environmental Quality. Letters confirming commitments from these agencies were appended. These state agencies agreed to play a role in collection of fish in the field. We also discussed our proposal with U. S. Fish and Wildlife Service (Dr. Charles Henry), and Oregon Cooperative Fish and Wildlife Research Unit (Dr. Carl Schreck) leadership. There are plans for collaboration with these Federal agencies, especially with regard to data interpretation.

Strong working relationships and complimentary expertise developed during conduct of the proposed research can lead to future joint proposals.

F. Expected Outcomes and Deliverables

One major outcome of this research will be peer-reviewed publications. Since the work is largely field-based, two complete field seasons of data are necessary for a quality publication. Laboratory bioassay results are best reported as separate publications. Preliminary results will be presented at regional and national scientific meetings. Since there is significant public interest in this issue, it may be useful to present some of this information in a public meeting. The legislature may expect reports. Finally, a written preliminary report will be available in December 2002 and the final written report will be completed by June 30, 2004.

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