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Article

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Environmental Stresses and Skeletal Deformities in Fish from the Willamette River, Oregon

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The Willamette River, one of 14 American Heritage Rivers, flows through the most densely populated and agriculturally productive region of Oregon. Previous biological monitoring of the Willamette River detected elevated frequencies of skeletal deformities in fish from certain areas of the lower (Newberg pool [NP], rivermile [RM] 26−55) and middle (Wheatland Ferry [WF], RM 72−74) river, relative to those in the upper river (Corvallis [CV], RM 125−138). The objective of this study was to determine the likely cause of these skeletal deformities. In 2002 and 2003, deformity loads in Willamette River fishes were 2−3 times greater at the NP and WF locations than at the CV location. There were some differences in water quality parameters between the NP and CV sites, but they did not readily explain the difference in deformity loads. Concentrations of bioavailable metals were below detection limits (0.6−1 μg/L). Concentrations of bioavailable polychlorinated biphenyls (PCBs) and chlorinated pesticides were generally below 0.25 ng/L. Concentrations of bioavailable polycyclic aromatic hydrocarbons were generally less than 5 ng/L. Concentrations of most persistent organic pollutants were below detection limits in ovary/oocyte tissue samples and sediments, and those that were detected were not significantly different among sites. Bioassay of Willamette River water extracts provided no evidence that unidentified compounds or the complex mixture of compounds present in the extracts could induce skeletal deformities in cyprinid fish. However, metacercariae of a digenean trematode were directly associated with a large percentage of deformities detected in two Willamette River fishes, and similar deformities were reproduced in laboratory fathead minnows exposed to cercariae extracted from Willamette River snails. Thus, the weight of evidence suggests that parasitic infection, not chemical contaminants, was the primary cause of skeletal deformities observed in Willamette River fish.

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Introduction

The Willamette River in western Oregon is one of 14 American Heritage Rivers and receives more runoff per square mile watershed than any other river in the U.S (*1*). It flows north for ∼187 miles through mixed agricultural and urban areas to Portland, Oregon's largest metropolitan area, before joining the Columbia River (Figure 1). The Willamette basin is home to 70% of Oregonians, and the Willamette Valley is one of the most highly productive agricultural regions in the Pacific Northwest (*2*−*3*). The Willamette River is a significant migratory corridor, nursery, and spawning habitat for salmon, and nearly 50 species of fish have been identified in the river (*3*). Recreational fishing is popular, and resident species are fished throughout the year.

In the early 1990s, the Oregon Department of Environmental Quality initiated investigations of skeletal deformities in Willamette River fishes. Biological monitoring has been widely used to evaluate aquatic ecosystem health and potential impacts of anthropogenic activities. It has been suggested that skeletal deformities in fish serve as a useful bioindicator of pollution (*4*−*6*), and evaluation of skeletal deformities in juvenile fish has been used to monitor the health of fish populations (*7*−*11*). In 1992−1994, the incidence of skeletal deformities in northern pikeminnow (*Ptychocheilus oregonensis*) collected from the Newberg (NP) region, extending from river mile (RM) 55 to 26.5 (Figure 1), ranged from 22 to 74% (*12*−*13*). Northern pikeminnow skeletal deformity rates were also elevated (21.7%) in the middle Willamette River (around RM 72, Wheatland Ferry; Figure 1). In contrast, the skeletal deformity rates in juvenile northern pikeminnow collected from the upper Willamette River (RM 185−125) ranged from 1.6 to 5.3% (*12*−*13*). Northern pikeminnow was not the only species impacted. Of 15 species collected from the Newberg region and associated tributaries in 2000, skeletal deformity rates exceeded 25% in 10 species (*14*). As a whole, biomonitoring of skeletal deformities in Willamette river fish suggested that fish from the Newberg region and middle Willamette River had significantly greater deformity rates than fish from the upper Willamette River.

In the mid-late 1990s, proposals to tap the Newberg region of the Willamette River as a source of drinking water for urban expansion heightened public concern related to the reports of deformed fish (http://www.hevanet.com/safewater/recentnewshome.htm). In 1998, for example, 85% of people surveyed expressed "extreme" concern about the level of toxic chemicals in the river (Oregon Daily Emerald, Feb. 26, 1998). This research was a response to such concerns and, especially, scientific uncertainty concerning potential causes.

A wide variety of chemical, physical, and biological stressors have been associated with skeletal deformities in fish. A variety of chemicals, including heavy metals, such as lead and numerous organophosphate pesticides, are known to induce neuromuscular damage that can result in skeletal deformities (*15*−*18*). Chemicals can also cause skeletal deformities by impairing developmental processes and bone formation. Compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polychlorinated biphenyls, toxaphene, and cadmium have been reported to cause skeletal deformities

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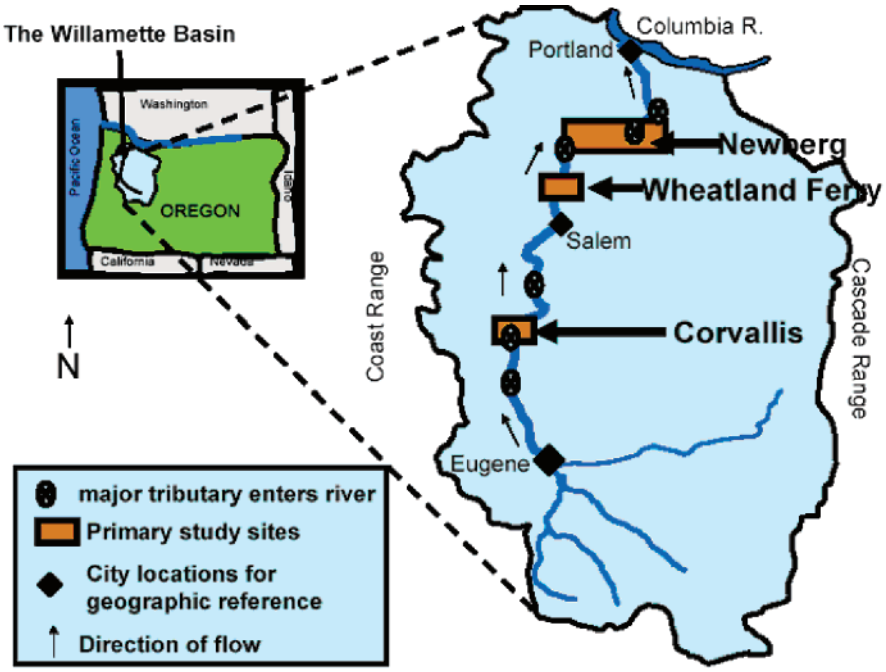

FIGURE 1. Diagram of the Willamette Basin depicting the general location and course of the Willamette River and primary study locations.

through such mechanisms (*19*−*24*). Skeletal deformities have also been linked to water quality problems, including low pH (*25*−*26*), low dissolved oxygen (*27*−*28*), and elevated temperatures (*29*−*30*). Nutritional deficits, particularly ascorbic acid and tryptophan deficiencies, have been linked to skeletal deformities in fish (*31*−*32*). Inbreeding has also been shown to cause skeletal deformities, including scoliosis, lordosis, curved neural spines, fused vertebrae, and compressed vertebrae (*33*−*35*). Finally, numerous infectious biological agents, including viruses, bacteria, and parasites, have been reported to cause skeletal deformities (*36*−*39*). Although the association of fish skeletal deformities with a wide variety of stressors makes it a useful endpoint for biological monitoring, the observation of a high incidence of skeletal deformities, alone, has little diagnostic value.

The purpose of this study was to identify or diagnose the cause(s) of skeletal deformities associated with Willamette River fish, with particular emphasis on the Newberg region. Skeletal deformities in fish collected from the upper, middle, and lower Willamette River in 2002 and 2003 were characterized to determine whether recent prevalences were similar to those reported previously and to further describe spatial and temporal patterns. In situ monitoring of river water quality coupled with in situ sampling and analysis of bioavailable organic contaminants and metals was used to compare water quality and potential for direct exposure to known chemical contaminants at the Newberg and Corvallis study sites and determine whether these factors were likely causes of the deformities. Analysis and comparison of sediment samples and fish tissue from Newberg and Corvallis was used to evaluate potential trophic or maternal transfer of known persistent organic pollutants (POPs) as a potential cause. Bioassay of river water extracts using embryo−larval fathead minnows (*Pimephales promelas*) exposed under controlled laboratory conditions was used to evaluate the potential role of unknown chemicals or complex chemical mixtures in causing the skeletal deformities observed in Willamette River fish. Field-collected fish were examined for parasites, and the association of parasitic infection with skeletal deformities was quantified. Finally, cercariae of a trematode parasite (identified as *Apophallus donicus)* were collected from Willamette River snails (*Fluminicola virens,* the intermediate host for *A. donicus*), and fathead minnows were exposed to the cercariae in the laboratory. Together, these components provided a weight-of-evidence-based, empirical approach to identify the likely cause of skeletal deformities in Willamette River fishes.

Methods

Fish Collection and Deformity Characterization. Larval and juvenile fish were sampled from May to October 2002 and May to August 2003. Fish were collected by beach seine, cast net, and dip net. The three primary sampling areas were Newberg pool (NP; RM 47.5−53; lower Willamette), Wheatland Ferry (WF; RM 72−74; middle Willamette), and Corvallis (RM 125−138; upper Willamette). Specimens were euthanized with an overdose of MS-222 (Finquel (tricaine methanesulfonate) Argent Chemical Laboratories, Redmond, WA at 500 ppm) and fixed in 10% buffered formalin. Seventeen species were collected, with *Ptychocheilus oregonensis* (northern pikeminnow), *Richardsonius balteatus* (redside shiner), *Catostomus macrocheilus* (largescale sucker), *Mylocheilus caurinus* (peamouth), and *Acrocheilus alutaceus* (chiselmouth) representing the most commonly collected species (total sample sizes > 1000) (*40*).

Specimens fixed for a minimum of two weeks were X-rayed in a Faxitron MX-20 cabinet X-ray machine using AGFA Structurix D4 DW ETE industrial radiography film. Film was developed using a Kodak X-OMAT model M6B developer. Radiographs of ~15 700 fish were inspected for deformities using a 10−15× ocular over a light table. Presence or absence of 12 different categories of skeletal deformities was scored (additional details in ref *40*), and our analyses were based on the number of deformity categories present per individual (deformity load) (*14*) plus the number of precaudal deformities, since caudal deformities are uniformly distributed (*40*). On the basis of random reevaluation of 550 fish, reader error was not significant (*40*).

In Situ Water Quality and Bioavailable Contaminants. *Site Description and Sample Collection.* Willamette River sampling sites were chosen to facilitate investigation of seasonal and spatial bioavailable contaminant concentrations at Newberg and Corvallis (Figure 1). Two sampling stations were located at Newberg, one on the south side of the river (RM 47; N 45°16.02′, W 122°54.59′) and one a few miles

downriver on the north side of the river (RM 44; N 45°15.27′, W 122°53.58′). Two stations were located at Corvallis (RM 135; [1] N 45°29.13′, W 122°39.06′; [2] N 45°27.37′, W 122°39.47′). Newberg station 1 (RM 47) was about 25−30 ft from the shoreline, and the local water depth was 27 ft. Newberg station 2 (RM44) was about 30 ft from the shore, and the local water depth was 20 ft. Both Corvallis stations were about 15 ft from the shore, and the local water depth was 7−11 ft. Flow near the Corvallis sites in May 2002 was ∼9000 to 10 000 ft^3/s and by late July had decreased to ∼4500 ft^3/s. At the Newberg sites, flow in May 2002 was ∼17 000 to 20 000 ft^3/s and by late July decreased to ∼7000 ft^3/s. The Corvallis area was generally shallow (2−12 ft) and characterized by shallow gravel and sediment riprap. In the Newberg area, the Willamette River was much deeper (20−60 ft), and there were no shallow gravel beds near the study sites. The Willamette River has very steep banks at the Newberg area: within 5−10 ft of the bank, the river is 15−20 ft deep. The bottom of the river in this area is a combination of rock and mud.

Water sampling was conducted from May to July in 2002 and 2003. Three 21-d sampling events were completed per year, one each in approximately May, June, and July. Sampling events were designed to characterize river conditions during spawning and early development. Nutrient and water quality parameters, including dissolved oxygen, specific conductance, salinity, total dissolved solids, temperature, pH, ORP (oxidation reduction potential), depth, ammonium, nitrate, and turbidity, were collected on an hourly basis with a YSI 6920 Sonde (YSI, Yellow Springs, OH).

Dissolved, bioavailable organic contaminants and metals were collected by deploying passive sampling devices (PSD) and diffusive gradient thinfilms (DGT) in protective mesh cages. PSDs consisted of neutral lipid (i.e., triolein) enclosed in layflat polymeric tubing (Environmental Sampling Technologies, St. Joseph, MO) (*41*). Five individual PSDs and one DGT (DGT Research Ltd, UK) were included in each cage. Each cage was suspended with a "float-cable-cage-cable-anchor" arrangement that ensured that the cage would stay at the station and would stay suspended one foot from the river bottom. The five PSDs were later composited for analysis. PSDs and DGTs were kept on ice in sealed, airtight, amber glass containers during transport to and from the field sites. Complete PSD descriptions have been published (*42*). PSDs were gently cleaned of sediment or algae after deployment at the site utilizing a tub filled with site water to minimize air exposure. No fouling impedance was employed in calculations of estimated water concentration, since algae growth on the devices was nil to minimal.

Analytical Procedure. PSDs were extracted by hexane dialyses, in amber glass jars. Sample volumes were reduced using a TurboVap LV (Zymark Corp. Hopkinton, MA). The samples were then run through gel permeation chromatograph (GPC) (models: 515 pump, 2487 dual wavelength absorbance detection, 717 auto-sampler, and fraction collector II, Waters, Corp. Milford MA), and fractions containing organochlorine pesticides, organophosphate pesticides, organonitrogen pesticides, PCBs, and PAHs were collected. The GPC columns were 19 mm × 300 mm divinylbenzene copolymer particles, 15-um particle size and 100-Å pore size. The GPC program ran with 100% dichloromethane at 5.0 mL/min. Appropriate fractions were determined by analyzing standards and fortified samples (*43*). Appropriate fractions were analyzed using GC-dual-ECD (organochlorines), GC-dual-NPD (organonitrogen and organophosphate pesticides), and HPLC−DAD and fluorescence (PAHs) (GC 6890N, Agilent Technologies, Pal Alto CA and HPLC 1100, Hewlett-Packard, Pal Alto CA). Sample manipulations were performed in either brown amber or foil-wrapped glass containers to minimize UV/vis exposure. Detailed analytical methods used for quantification of organochlorines, organonitrogen pesticides, and organophosphate pesticides are provided elsewhere (*43*).

The polycyclic aromatic hydrocarbons (PAH) contaminants fraction was separately concentrated to ∼1.0 mL. PAH detection and quantitation was performed on a HPLC with dual detection by flourescence or diode array, both with multiple wavelengths. The fluorescence detector had an excitation wavelength at 230 and emission wavelengths at 360, 410, and 460 nm; the diode array had detection signals at 254, 242, and 230 nm. Only three compounds, fluorene, acenaphthylene, and indeno(123*cd*)pyrene, were detected by diode array; the rest were detected with the fluorescence detector. The column used was a Phenomenex Luna C18, with 3-μm particle size. The instrument was run with a constant flow rate of 0.75 mL/min and a timed gradient for the acetonitrile/water eluent system. The time program ran at 40% acetonitrile for 10 min, was gradually ramped up to 70% acetonitrile for 15 min, and then ramped up to 90% acetonitrile for 10 min. The program was held at 90% acetonitrile for 3 min and then returned to 40% and analyzed by HPLC with diode array detection (DAD) and fluorescence detection. The approximate retention times in min are naphthalene, 16.0; acenaphthylene, 16.9; fluorene, 18.1; phenanthrene, 18.15; anthracene, 18.6; fluoranthene, 19.0; pyrene, 20.0; chrysene, 20.4; benzo(*a*)anthracene, 22.1; benzo(*b*)fluoranthene, 24.5; benzo(*k*)fluoranthene, 24.8; benzo(*a*)pyrene, 25.1; dibenzo(*a*,*h*)anthracene, 27; benzo(*g*,*h*,*i*)-perylene, 28.8; and indo(1,23,*cd*)pyrene, 29.2.

After DGTs were retrieved and in the laboratory, the resin-gel was removed and immersed for 24 h in 1.0 mL of 1 M trace metal grade nitric acid (Fisher Scientific). Acetic acid and sodium acetate were used as the supporting electrolyte, and the samples were diluted to a final volume of 25 mL with 18-MΩ·cm water. The analysis was by anodic stripping voltammetry (ASV) (TraceDetect, Seattle, WA). All grab water samples were filtered thru a 0.45-μm membrane filter prior to metal analyses by ASV. ASV was used to quantify the metals reported. Reduction potentials were verified with standards for each metal tested.

Quality Control (QC). Field, trip, and extraction blanks were used with each sampling event. Field blank PSD samples were opened and exposed to the atmosphere during deployment or recovery. Field blanks were processed and analyzed exactly as deployed PSD samplers. Field extraction blanks were opened in the field and washed simulating the process of removing the light sediment or algae on the passive sampling devices. Samples containing residues exceeding the blanks were considered positive for residues. Transport blank values were multiplied by the water volume they would have been exposed to if left with the other PSDs. The Corvallis site was designated as a field duplicate site. Field duplicates represented 30% of all samples collected. All QC sample types were included in each analytical batch. Laboratory QC samples included reagent blanks, fortified samples, and laboratory duplicates. Each QC type represented 5−10% of the total number of samples analyzed in any given batch. They were prepared and analyzed in the same fashion as the field samples. Organic standard (ChemService, West Chester, PA) curves were typically composed of ≥4 standard concentrations and metal standard (Alfa Aesar, Ward Hill, MA) curves ≥3 for all analyses.

Data Analysis. The theory and mathematical models required for estimation of analyte water concentrations from the concentration in the PSD lipid have been described (*42*). The following equation was used to calculate the dissolved (bioavailable) water concentration,

$$C_w = C_{SPMD} M_{SPMD} / R_s t$$

where C_w is the concentration of analyte in water, C_{SPMD} is

the concentration in lipid (SPMD), t is the exposure time in days, M_{SPMD} is the mass of SPMD in g, and R_s is the PSD sampling rate. Sampling rates (R_s) for a large series of OC and PAH contaminants have been previously established.

The mass of the metal in the DGT resin gel (M) was determined from the ASV quantitation. The theory and mathematical models required for estimation of the analyte water concentrations from the concentration in the DGT have been previously described (*44*). The following equation was used to calculate the labile (bioavailable) water concentration,

$$M = C_e(V_{HNO_3} + V_{gel})/f_e$$

where C_e was the concentration of metals in the 1 M HNO_3 elution solution, V_{HNO_3} was the volume of HNO_3 added to the resin gel, V_{gel} was the volume of the resin gel, and f_e was the elution factor for each metal. The concentration of the metal measured by DGT (C_{DGT} = "bioavailable" water concentration) was determined from the following equation,

$$C_{DGT} = M\Delta g/(DtA)$$

where Δg was the thickness of the diffusive gel (0.8 mm) plus the thickness of the filter membrane (0.13 mm), D was the diffusion coefficient of metal in the gel, t was deployment time, and A was the exposure area (A = 3.14 cm^2) (*44*).

Analysis of POPs in Northern Pikeminnow Ovary Tissue. Northern pikeminnow (*P. oregonensis*) was the species chosen for analysis of maternal transfer of POPs. They are abundant at both study locations, relatively easy to collect, reach moderately large sizes, and have large number of deformities in the Newberg region (*12*, *14*). Adults were collected from Newberg (N 45°16.007′, W 122°55.031′) and Corvallis (N 44°28.250′, W 123°14.300′) (Figure 1) in May−June 2002 using a combination of hook and line and electrofishing and transported to the laboratory on ice. Wet weights ranged from 375 to 975 g, and there was no significant difference in the mean wet weight of the fish collected from the two study sites. Ovarian tissue and associated oocytes were removed from gravid females using clean, solvent-rinsed, dissection tools; placed into certified I-Chem jars; and stored at −20 °C until extracted.

Samples were shipped to GLP (Good Laboratory Practices)-certified analytical laboratories for quantification of a range of POPs. Twenty-one chlorinated pesticides were quantified by gas chromatography with electron capture detection (GC/ECD) according to EPA method 8081A (ODEQ laboratory, Portland OR). Twenty-eight polychlorinated biphenyl (PCB) congeners were quantified by GC/ECD according to EPA method 8082 (ODEQ laboratory, Portland, OR). Additionally, concentrations of seven polychlorinated dibenzo-*p*-dioxin (PCDD) congeners and 10 polychlorinated dibenzofurans (PCDFs) were quantified by high-resolution GC/MS (Axys Analytical, British Columbia, Canada). Method detection limits (MDLs) for chlorinated pesticides and PCBs ranged from 2.5 to 3.3 μg/Kg wet wt. MDLs for PCDDs and PCDFs ranged from 0.1 to 0.13 ng/Kg wet wt.

Five ovarian tissue/oocyte samples (each from a separate fish) were analyzed per study area. For statistical analysis and plotting of figures, concentrations below the method reporting limit (MRL) or detection limit were assumed to be equal to one-half of the limit. When assumptions of parametric statistics were met, *t*-tests were used to test for differences among study sites. Kolmogorov−Smirnov's test was used in cases that parametric assumptions were not met.

Analysis of POPs in Sediment. Grab samples of surficial sediment were collected from Newberg and Corvallis sites. In 2002, three samples were collected at Newberg location N 45°16.308′, W 122°59.460′, and three samples were collected at Corvallis location N 44°31.567′, W 127°15.384′. In 2003, three samples were collected at Newberg location N 45°15.567′, W 122°54.231′, and three samples were collected at Corvallis location N 44°32.887′, W 123°15.432′. Sediment samples were scooped directly into certified I-Chem jars, transported on ice to the laboratory, and stored at −20 °C until shipped for analysis. Sediment samples were extracted and analyzed for 22 chlorinated pesticides by GC/ECD (EPA method 8081A), 8 nitrogen/phosphorus pesticides by GC/NPD, and 29 PCB congeners by GC/ECD (EPA method 8082 A) at the ODEQ laboratory, Portland, OR. MDLs for chlorinated pesticides and PCBs were ∼0.33 μg/Kg wet wt. The MDL for nitrogen/phosphorus pesticides was 10 μg/Kg wet wt.

Skeletal Deformities Bioassay I. River Water Extracts. Water samples were collected from four study locations during the summer of 2003. Sampling sites included two Newberg locations (NP: N 45°15.567′, W 122°59.142′ and AI N 45°16.145′, W 122°59.142′), Wheatland Ferry (WF: N 45°05.447′, W 123°02.655′), and Corvallis (CV: N 44°32.887′, W 123°15.432′). On each sampling day, samples were collected from CV and one of the other three sampling locations. At each site, three 20-L grab samples were collected in stainless steel containers. Samples were typically collected at a depth of ∼1 m, and containers were opened and sealed (all air removed) underwater. In all cases, collections were made at least 30 cm below the surface and at least 30 cm above the sediment. Sample extraction was completed within 96 h of sample collection.

The 60 L of water collected at each site (triplicate 20 L samples) was divided into five 12 L subsamples for extraction. Each subsample was filtered under vacuum through a 50-mm DVB-phobic followed by a DVB-phillic solid-phase extraction disk (Bakerbond Speedisk 8072-06, 8068-06, J. T. Baker, Phillipsburg, NJ). Flow rates were 15−30 mL/min. Following extraction, the disks were dried under vacuum and stored in airtight containers at −20 °C overnight. To prepare bioassay concentrates, each disk was eluted three times with 5 mL of methanol. Methanol eluents were dried by passing through a column of Na_2SO_4. For each site, dried methanol eluents were pooled and evaporated to 3 mL under a steady stream of N_2 gas using a Zymark Turbovap II. The pooled concentrates were transferred to amber glass vials and stored at −80 °C until used for bioassay.

Although not as exhaustive as multimethod procedures designed for the extraction and analysis of a wide range of organic contaminants in surface water (*45*), the extraction procedure described above was designed to capture a significant cross section of dissolved organic contaminants (log K_{ow}'s in the range of 1−7), with a resulting methanol concentrate suitable for use in a fathead minnow bioassay. Using the extraction procedure described above with ethyl acetate as the eluent, dissolved residues of over 100 current-use pesticides and POPs have been recovered and analyzed by capillary GC/MS (Usenko and Simonich, personal communication). To assess extraction efficiency, river water collected at the CV site was fortified at 0.0075 μg/L with chlorpyrifos (log K_{ow} = 4.7), a well-known Willamette River contaminant. Ethyl acetate extracts were analyzed by GC/MS using the method of Usenko and Simonich. Average recovery ± standard deviation was 91 ± 5 (n = 9).

Fathead minnows (*P. promelas*) less than 24 h post-hatch were obtained from Chesapeake Cultures (Hayes, VA). Larval fathead minnows (FHM; 24−48 h post-hatch) were randomly assigned to 400-mL beakers containing 100 mL of dechlorinated tap water (dtw). Each beaker was stocked with n = 30 larval FHM. Beakers were then randomly assigned to one of eight treatment groups. Treatment groups for the study wer: control (CON; 200 mL of dtw); solvent control (SC; 0.05%

MeOH in dtw); 8X-, 4X-, and 1X-Corvallis; 8X-, 4X-, and 1X-NP, AI, or WF. 8X, 4X, and 1X represent the volume of the appropriate extract dissolved in 200 mL dtw to provide a concentration equivalent to 800, 400, and 100%, respectively, of river water concentration of the extract's constituents, assuming 100% recoveries. Methanol was added to each of the 4X and 1X treatments such that the total MeOH concentration was equivalent to that of the 8X treatments and SC (0.05%). Fifty percent of the test solution was renewed daily by drawing the solution down to 100 mL and adding 100 mL of fresh test solution containing nominal concentrations of extract, solvent, or both. The location of each beaker on the exposure bench was assigned randomly, and all beakers were aerated throughout the exposure duration.

After 5 days of exposure, surviving fish were counted and transferred to 1-L plastic containers for grow-out to ~d hb 25−30 post-hatch. During grow-out, fish were maintained in dtw supplied from a flow-through system. Throughout both exposure and grow-out, water temperatures were maintained at 24−26 °C, photoperiod was 16 h light, 8 h dark, and FHM were fed *Spirulina* (Algae Feast, Earthrise, Petaluma, CA) twice daily and brine shrimp nauplii (GSL Brine Shrimp, Ogden, UT) once daily. Dissolved oxygen, pH, ammonia, and nitrite were monitored daily.

At the end of the grow-out period, fish from each container were transferred to a 5-cm-diameter plastic tube with fine mesh at one end (PVC insert). The entire batch of live fish was immersed in a 0.2% calcein (Sigma C-0875; St. Louis, MO) solution (pH 7.0), stained for 10 min, transferred to clean water for 10 min to destain, and then euthanized by immersion in a 200 mg/L solution of MS-222 (Finquel, Argent, Redmond, WA). Euthanized specimens were immediately examined by fluorescence microscopy using a Leica MZFL111 dissecting microscope (Bartles and Stout, Bellevue, WA) equipped with a mercury lamp and fluorescein/green fluorescence protein filter. Calcein staining allows for direct visualization of calcified skeletal structures (*46*). Each specimen was examined for skeletal deformities, including scoliosis, lordosis, fused vertebrae, compressed centra, extra or missing spines, etc. Screening of several hundred fish as part of assay development confirmed that all these types of deformities were detectable by this method. Vertebral development was also scored on a scale of 1−5 using a criteria defined for this study. Digital images of each fish and close-ups of deformities, if detected, were captured and archived using ImagePro Plus 4.5.1 (Media Cybernetics, Silver Springs, MD). In some cases, examinations were spread over 2−3 d. Replicates examined each day were selected randomly.

Survival to the end of exposure (6 d post-hatch), survival to examination (28−30 d post-hatch), and percent of surviving fish with a skeletal deformity were determined for each replicate. Developmental score distributions were determined for each treatment. One-way analysis of variance was used to test for differences in survival or incidence of deformities among treatments. A nonparametric Kruskall−Wallis test on ranks was used to test treatment-related differences in developmental score distributions.

Skeletal Deformities Bioassay II. Exposure to *Apophallus donicus* Cercariae. Characterization of parasite association with vertebral deformities in Willamette River fishes was based on examination of histological sections of formalin-preserved fish as well as whole mounts of trypsin-cleared, alcian blue and alizarin red S-stained fish (*47*). The methods and statistical analysis used for the parasite characterization were reported elsewhere (*47*).

For laboratory transmission studies, laboratory-reared fathead minnows were obtained from Cheasapeake Cultures, Hayes, VA. Fish were held in dechlorinated tap water (23−26 °C) to ensure unexposed fish did not become infected. Fish were maintained in static water aquaria with biological filters. Fish were delivered at 3−7 day old and were initially fed paramecium cultures until about 10−14 days old, then were switched to a mixture of brine shrimp naupallii (GSL Brine Shrimp) and freeze-dried Spirulina algae (Algae Feast). After about 3−4 weeks, fish were then fed TetraMin flake food (Tetra Sales, Blacksburg, VA).

Fluminicola virens snails were collected from the Newberg area (Figure 1) from June to August 2003. Cercariae consistent with those described by Niemi and Macy (48), (Figure 2a) were harvested from individual snails by holding snails in isolation in 24-well tissue culture plates in 2 mL of water. For transmission studies, larval fathead minnows of varying age (Table 4) were exposed to known concentrations of cercariae or control water. Initial trials with very young fish resulted in high mortality in exposed fish (Table 4).

Incidence of infection, vertebral deformities, and association of worms with deformities was determined by examination of whole, preserved fish that were cleared with trypsin and stained with with alcian blue and alizarin-red S (*49*−*50*). Fish were collected at either 55 or 70 days postexposure. Cleared fish were placed in a Petri dish, covered with glycerin, and examined at 25 or 50×. Fish were also evaluated by radiography, as described by Markle et al. (*14*).

Results and Discussion

Deformity Loads in Willamette River Fish. One difficulty associated with the use of fish skeletal deformities as a biomonitoring tool is the lack of information on normal background deformity rates. One study in salmonids suggested that 2−5% may be a normal background rate in wild populations (*51*); however, it is unclear whether deformity rates differ among species or among populations within species. Because background deformity rates are usually unknown, biomonitoring approaches based on skeletal deformities rely on temporal or spatial comparisons, such as year to year changes, or comparisons between locations with similar habitat, climate, etc. In Willamette River fish, the marked geographic disparity in frequency of skeletal deformities suggested localized problems at Newberg and Wheatland Ferry.

Our 2002−2003 results were consistent with previous studies that reported a greater incidence of skeletal deformities in Willamette River fish from Newberg and Wheatland Ferry relative to Corvallis (*12*, *14*). Among the five species most commonly sampled, percent frequency of deformities was generally 2−3 times greater at Newberg and Wheatland Ferry than at Corvallis (Table 1). The only exception was the large-scale sucker (*Catostomus macrocheilus*), which was the only catostomid of the five most commonly collected fishes. Among the cyprinids, mean deformity loads were usually significantly lower for Corvallis fish than for fish from Wheatland Ferry or Newberg (Table 1). There were no obvious geographic or habitat differences that explained the differences observed (*40*). Overall, biomonitoring of skeletal deformities in fish collected at different locations along the Willamette River in 2002−2003 supported the conclusion that fish populations near Newberg and Wheatland Ferry were more likely to have skeletal deformities than fish from Corvallis. Spot historical samples from museum collections confirm high precaudal deformity loads (0.33−1.54 per fish) in 1983 in Newberg and Wheatland Ferry, a lower load in 1952 (0.12) at Wheatland Ferry, and a lower load upstream of Corvallis in 1967 (0.12) (*14*). These historical data do not help us distinguish among three possibilities: (1) site variation, and the range of variation we detect is normal; (2) rates at Corvallis represent the background, and Newberg rates are elevated; and (3) rates at Newberg represent the background, and Corvallis rates are depressed.

Water Quality Characterization. Ammonia, nitrate, pH, temperature, dissolved oxygen, oxidative reduction potential,

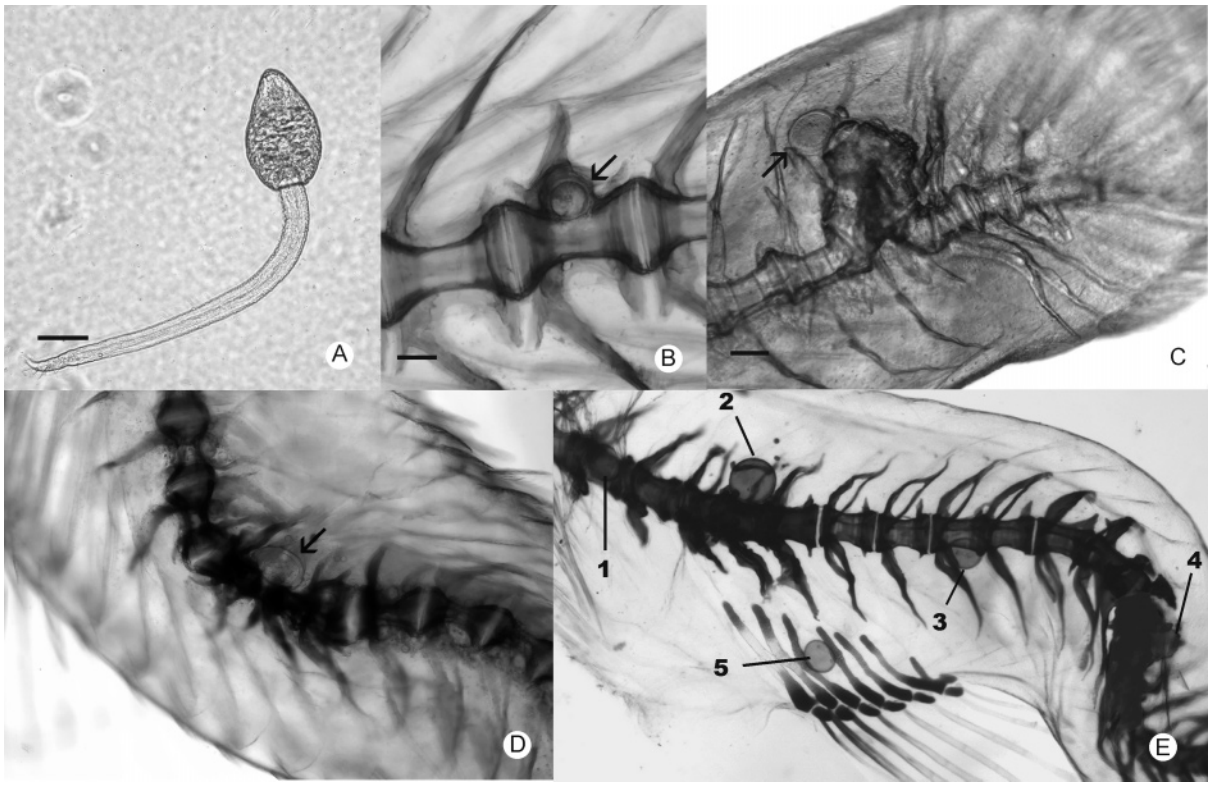

FIGURE 2. ***A. donicus*** **infections in laboratory-reared fathead minnows. A.** ***Paraplophocercous*** **cercaria from** ***F. virens*** **used in exposure trials. Bar = 50 μm. B−E. Metacercariae (arrows) associated with skeletal deformities in cleared fish. Bar = 200 μm. B. Metacercariae in surrounded by bony proliferation at base of a vertebra. C. Metacercaria in region of severe lordosis. D. Dysplastic vertebrae and lordosis at site of infection. E. Metacercariae associated with lesions: 1 and 3, no significant changes; 2, dysplastic and broken spines; 4, severe lordosis; 5, metacercariae in base of anal fin with no changes.**

TABLE 1. Frequency of Occurrence of Precaudal Deformities and Average Deformity Loads for the Five Species Most Commonly Collected from Three Willamette River Study Areas in 2002−2003

	Newberg pool			Wheatland Ferry			Corvallis		
species	%[a]	DL[b,c]	N[d]	%[a]	DL[b,c]	N[d]	%[a]	DL[b,c]	N[d]
Ptychocheilus oregonensis, northern pikeminnow	23.6	0.37 ± 0.016, A	2314	22.8	0.35 ± 0.021, A	1205	6.9	0.08 ± 0.01, B	928
Richardsonius balteatus, redside shiner	14.2	0.24 ± 0.029, A	515	13.0	0.20 ± 0.018, A	1091	6.3	0.09 ± 0.020, B	349
Catostomous macrocheilus, largescale sucker	14.8	0.21 ± 0.015, A	1394	21.3	0.34 ± 0.111, A	47	17.3	0.22 ± 0.050, A	127
Mylocheilus caurinus, peamouth	14.1	0.20 ± 0.032, AB	305	24.2	0.34 ± 0.033, B	442	8.4	0.10 ± 0.038, A	96
Acrocheilus alutaceus, chiselmouth	39.6	0.70 ± 0.061, A	268	55.1	0.97 ± 0.103, B	109	12.8	0.17 ± 0.030, C	251

[a] Frequency of occurrence of precaudal deformities. [b] Deformity load; number of deformity categories present per individual; mean ± SE (SE is individual rather than pooled). [c] A,B,C indicate significant difference between sites ($p \leq 0.05$) based on Bonferroni multiple range test. [d] Sample size.

and specific conductance data were collected hourly during all 21-d sampling events. At each station, some parameters showed strong temporal and spatial variation, while others did not. Diurnal temperature variation was greater at the Corvallis sites (1−2 °C), than at Newberg (1 °C or less). Temperature patterns were the same for both years and were generally 12 ± 1 °C in May and rose to ~22C ± 2 °C by the end of the sampling period. Maximum pH range during 24 h, usually in late May to early June was 7.2 to 7.8 at the Newberg sites and 7.2 to 8.8 at the Corvallis sites. These large diurnal pH changes were seen in both years. At all sites, there were small decreases in pH from 2002 to 2003. Nighttime pHs were similar, but the daytime pH showed a strong geographic difference, with the Corvallis sites often 1+ pH units higher than the Newberg sites. Low pH conditions (<5.5) have been linked to skeletal deformities in suckers (*25*−*26*), but we are not aware of any reports that link pH 7.2−8.8 conditions to skeletal deformities in fish.

The oxidation reduction potential (ORP) probe tended to drift after about 7−10 days of deployment. Since all deployments were 21 days, data after 7 days were excluded from the analysis. ORP was consistently lower at the Newberg sites, as compared to the Corvallis sites. The difference ranged from <10% to a factor of 2. There was no apparent seasonality at the Corvallis sites and little difference between 2002 and 2003. There was some evidence that the ORP increased during the season and differed among years at the Newberg sites. The ORP value is a direct reading of the activity of oxidizing and reducing agents in the water, as they correspond to oxidation−reduction reactions (*52*). In general, the Corvallis

TABLE 2. Mean Concentrations (ng/L) of Bioavailable Organics Estimated from Concentrations Accumulated in Passive Sampling Devices Exposed for 21 d at Two Sites (2 locations per site) along the Willamette River[a]

	Corvallis		Newberg pool	
bioavailable	2002	2003	2002	2003
Σ PAH[c]	3.17 ± 1.47	3.22 ± 1.22	2.01 ± 0.34	1.72 ± 0.54
phenanthrene	1.15 ± 0.60	0.58 ± 0.22	0.93 ± 0.27	0.375 ± 0.107
anthracene[b]	1.32 ± 0.52	1.18 ± 0.90	0.43 ± 0.12	0.04 ± 0.02
fluoranthene[d]	0.70 ± .40	0.43 ± 0.13	0.66 ± 0.07	0.29 ± 0.07
Σ PCB[d]	0.007 ± 0.011	0.009 ± 0.004	0.013 ± 0.015	0.015. ± 0.005
Σ DDT	0.022 ± 0.028	0.036 ± 0.004	0.049 ± 0.018	0.048 ± 0.011
p,p′-DDT[c]	0.003 ± 0.005	0.011 ± 0.001	0.010 ± 0.005	0.011 ± 0.002
p,p′-DDE[c]	0.006 ± 0.008	0.016 ± 0.003	0.016 ± 0.005	0.021 ± 0.006
p,p′-DDD[d]	0.013 ± 0.019	0.010 ± 0.002	0.023 ± 0.005	0.015 ± 0.004
dieldrin[b]	0.008 ± 0.009	0.014 ± 0.002	0.023 ± 0.007	0.025 ± 0.006
chlorpyrifos	NA	0.74 ± 0.51	NA	1.38 ± 0.33

[a] Target analytes with concentrations < detection limit not shown. See Supporting Information for complete list of analytes. [b] Significant difference between sites, both years. [c] Significant difference between sites, 2002 only. [d] Significant difference between sites, 2003 only.

TABLE 3. Concentrations of Chlorinated Pesticides, Polychlorinated Biphenyls, Polychlorinated Dibenzo-*p*-dioxins, and Polychlorinated Dibenzofurans Detected in Oocyte/Ovary Tissue from Northern Pikeminnow (*Ptychocheilus orgeonensis*) Collected from Newberg Pool (NP) and Corvallis (CV) Study Sites

	mean ± SE (ng/g)[a,b]		median (ng/g)[a]		
compound	NP	CV	NP	CV	P
endrin	3.94 ± 1.62	*1.62* ± 0.01	1.65	1.62	0.191
4,4′-DDD	3.63 ± 1.34	*1.62* ± 0.01	1.65	1.62	0.171
4,4′-DDE	46.9 ± 13.4	48.0 ± 15.9	35.0	31.0	0.942
PCB-8 [2,4′]	3.26 ± 1.74	10.8 ± 4.68	1.65	7.70	0.144
PCB-18 [2,2′,5]	*1.55* ± 0.07	16.7 ± 15.1	1.65	1.65	0.999
PCB-101 [2,2′,4,5,5′]	3.52 ± 0.90	*2.21* ± 0.06	4.16	1.65	0.402
PCB-110 [2,3,3′,4′,6]	*1.98* ± 0.46	*1.62* ± 0.01	1.65	1.62	0.674
PCB-118 [2,3′,4,4′,5]	*2.48* ± 0.96	*1.62* ± 0.01	1.65	1.62	0.674
PCB-138 [2,2′,3,4,4′,5′]	*2.80* ± 1.27	*1.62* ± 0.01	1.65	1.62	0.674
PCB-153 [2,2′,4,4′,5,5′]	4.20 ± 0.92	*2.65* ± 0.65	4.20	1.65	0.163
$TEQ_{[PCDD/DFs]}$ (pg/g wet wt)	0.84 ± 0.20	0.85 ± 0.31	0.99	0.67	0.959

[a] For the purposes of calculating means, medians, and statistics, nondetects were assumed to be equal to 1/2 the method detection limit. All concentrations reported in ng/g wet wt, except TEQ, which are reported as pg/g wet wt. [b] *Italics* indicates that mean or median estimate was less than the MDL.

TABLE 4. Incidence of Vertebral Deformities and Metacercariae in Fathead Minnows (Pimephales promelas) Exposed to Cercariae of *Apophallus donicus*

trial no.	concn of exposure cercariae/fish	age of exposure (days)	days postexposure when examined	number examined	deformed %	infected %	abundance	deformities associated with parasites, %	worms associated with deformities, %
1	30	8	55	11	8/11 (73)	9/11 (82)	1.0	9/10 (90)	9/11 (82)
1C	0	8	55	7	0	0	0	NA	NA
2	10	8	70	14	12/14 (86)	13/14 (93)	1.9	17/17 (100)	20/27 (74)
2C	0	8	70	18	1/18 (6)	0	0	0	NA
3	30	5	70	10	7/10 (70)	8/10 (80)	1.2	8/9 (88)	8/12 (67)
3C	0	5	70	12	0	0	0	NA	NA
4	30	17	70	14	13/14 (93)	14/14 (100)	4.5	31/33 (94)	38/64 (59)
5	30	24	70	21	19/21 (91)	20/21 (95)	4.0	37/42 (88)	58/88 (66)
4/5C	0	17/24	70	18	1/18 (6)	0	0	0	NA
	total exposed			70	84	91	2.5	93	66
	total controls			55	4	0	0	0	0

waters were more oxidizing, or less reducing, than Newberg waters. ORP is known to influence microbial growth, but it is not clear whether the difference in ORP observed would make Newberg fish more vulnerable to infection by microbial agents or more susceptible to deformities.

Specific conductivity (SC) was very similar at the Corvallis and Newberg sites, with both showing a slight increase from May to July. The SC pattern was similar for both years. The dissolved oxygen (DO) pattern was similar at both sites. A diurnal pattern was apparent, and a slight decrease in DO was seen from May to July in both 2002 and 2003. A seasonal increase in ammonia from 0.05 to 0.25 mg/L was measured at both sites, with Corvallis generally having higher ammonia than Newberg. The nitrate probe was not robust. In 2002, drift generally occurred within 24 h of deployment. In 2003, the probe failed within a few hours of deployment. The limited data indicated that nitrate increased from May to July, and concentrations were higher at the Newberg sites, as compared to the Corvallis sites. As a whole, water quality monitoring provided no compelling evidence to suggest that differences in nutrient concentrations, pH, temperature, DO, or specific conductivity were likely causes of the different deformity

loads observed at Newberg versus Corvallis, but further investigation of a possible link between ORP differences and susceptibility to infection may be warranted.

Bioavailable Contaminants. *Polycyclic Aromatic Hydrocarbons (PAHs).* Total bioavailable PAHs were low at all locations and for all sampling events (generally <4 ng/L). Of the 16 PAHs measured, 13 PAHs were below detection limits (<0.1 ng/L) in 2002, and 10 PAHs were below in 2003. Three PAHs—phenanthrene, fluoranthene, and anthracene—were detected at sites in 2002 and 2003. At the Corvallis sites, phenanthrene and anthracene were equally abundant in 2002, whereas fluoranthene was somewhat less abundant (Table 2). Of the three PAHs detected at the Newberg sites in 2002, phenanthrene was the most abundant (Table 2). In 2003, anthracene was most abundant at Corvallis, and phenanthrene was most abundant at Newberg of the 16 individual bioavailable PAHs. In 2002, the Corvallis sites consistently had higher total PAH (ΣPAH) concentrations, as compared to the Newberg sites (Table 2). The average ΣPAH in 2002 was ∼3.2 ng/L at Corvallis, whereas the Newberg sites had ΣPAH concentrations in 2002 of about ≤2 ng/L. In 2003, this pattern was again upheld (Table 3). The Corvallis stations (1 and 2) had ΣPAHs of 3.9 and 2.7 ng/L respectively; the Newberg stations (1 and 2) had ΣPAHs of ≤2 ng/L. Phenanthrene produces spinal curvature in zebrafish embryos at concentrations near water saturation (1.25 mg/L) (*53*). With low concentrations detected at both sites, PAHs were not considered to be likely contributors to the difference in deformity loads associated with the two areas.

Polychlorinated Biphenyls (PCBs). PCB analysis for this study was based on a congener-specific approach; however, concentrations of all congeners were quite low, so interpretation of results focused on total PCB concentrations. The total bioavailable PCB concentrations were generally very low, <0.03 ng/L, at all sites, and many sites were below detection limits (0.001 ng/L) (Table 2). During 2002 and 2003, total bioavailable PCBs were generally greater at the Newberg sites (Table 2). However, since most of the data were below or near detection limits, it was difficult to draw any firm conclusions. PCB concentrations were approximately 500-fold lower than the chronic ambient water quality criteria, (CCC) of 0.014 μg/L (*54*) and they did not readily explain the difference in deformity loads among sites.

Pesticides. The bioavailable ΣDDT (sum of *p′p*-DDT, *p,p′*-DDD, and *p,p′*-DDE) concentrations were low (generally <0.07 ng/L) at all sites in 2002 and 2003 (Table 2). ΣDDT concentrations were ∼10-fold below the freshwater CCC, 0.001 μg/L (*54*). The differences in ΣDDT between Newberg and Corvallis sites in 2002 ($p = 0.18$) and in 2003 ($p = 0.065$) were not significant (Table 2). Interannual differences in ΣDDT among stations were not significant ($p = 0.49$ Newberg; $p = 0.24$ Corvallis). In 2002, the DDT profile was dominated by *p,p*-DDD, followed by DDE and DDT (Table 2), as would be expected from older deposits. However, in 2003, DDE dominated, followed by DDD and DDT (Table 2). DDD and DDE concentrations detected at Newberg sites in 2002 and 2003 were not significantly different ($p = 0.18$). DDE concentrations but not DDD concentrations at Corvallis sites increased significantly between 2002 and 2003 ($p = 0.041$ DDE; $p = 1$ DDD). Mean DDT concentrations at Newberg did not increase significantly between 2002 and 2003 ($p = 1$; Table 2), but mean bioavailable DDT detected at Corvallis increased 4-fold ($p = 0.004$; Table 2). The cause of the increased *p,p′*-DDT concentration was not clear, but the results suggest possible new inputs or sediment disruption remobilizing DDT. Overall, the concentration differences observed were very small (≈0.008 ng/L), so conclusions should be drawn prudently.

Dieldrin concentrations were slightly higher at the Newberg sites, as compared to the Corvallis sites (Table 2; 2002 $p = 0.015$; 2003 $p = 0.004$). However, the bioavailable concentrations of dieldrin were very low, <0.03 ng/L, and there was no significant difference between years (Newberg $p = 0.394$; Corvallis $p = 0.818$; Table 2). The dieldrin concentrations detected were around 2000-fold below the freshwater CCC of 0.056 μg/L (*54*) and are well below those generally thought to be toxic to fish (*55*−*56*). We are not aware of any reports linking below nanogram-per-liter concentrations of dieldrin to skeletal deformities.

Chlorpyrifos was the only organophosphate pesticide detected. Dimethoate, diazinon, and azinphos-methyl were below detection limits at all sites for all sampling events (detection limits were estimated at 2, 3, and 2 ng/L, respectively). In 2003, the estimated, bioavailable, water concentration for chlorpyrifos averaged 0.74 ± 0.51 at Corvallis and 1.38 ± 0.33 ng/L at Newberg (Table 2). There was little difference between the sites in June and July, but there was some difference between the sites in May, with chlorpyrifos concentrations at the Newberg sites higher than at the Corvallis sites. However, the difference was not significant ($p = 0.33$). The bioavailable chlorpyrifos concentrations observed in this study were below the chronic aquatic life criteria of 0.04 μg/L and well below concentrations reported to be toxic to fish (*57*). Observed chlorpyrifos concentrations are also well below the 95% confidence lower limit of the benchmark concentration estimate of 0.015 μg/L resulting in 2.5% brain acetylcholinesterase (AChE) inhibition in steelhead trout (*58*). Brain AChE is considered as a sensitive indicator of sublethal effects. Additionally, we are unaware of any peer-reviewed reports linking lesser concentrations of chlorpyrifos to skeletal deformities in fish.

Metals. Bioavailable heavy metal samples were collected using diffusive gel thin films (DGT), and samples were deployed in the same manner as the other PSDs. Bioavailable zinc, cadmium, lead, and copper were determined for all six sampling events. Zinc, cadmium, lead, copper, and arsenic (III) were determined in filtered grab water samples pulled during the 2003 sampling deployments. The bioavailable metal concentrations were, for the most part, below detection limits. The only exceptions were detection of ∼100 μg/L of Zn in a single sample from Newberg and 7 μg/L of Pb in a single sample from Corvallis. Neither of these detections were replicated, and all other samples from both sites were below detection limits, so both detections were considered artifacts. The results provided no evidence that bioavailable heavy metals were a likely cause for the difference in deformity loads at the two sites.

Maternal Transfer of POPs. Concentrations of POPs detected in Willamette River northern pikeminnow ovary/oocyte tissue were relatively low. Chlorinated pesticide concentrations were generally <3.3 ng/g wet wt, and only 3 of the 21 different chlorinated pesticide residues analyzed were detected (Table 3). Of the three, 4,4′-DDE was detected with the greatest frequency and at the greatest concentrations (Table 3). Endrin and 4,4′-DDD were detected in 2 of 10 samples analyzed, both from Newberg fish. Exposure to parts-per-billion concentrations of toxic chlorinated pesticides similar to those detected in the ovary/oocyte tissue from some of the fish analyzed have been shown to cause adverse effects in early life stage fish (*59*−*61*). Thus, some potential for toxic effects of maternally transferred chlorinated pesticides was possible, although more extensive study would be needed to determine how probable such effects are.

Concentrations of maternally transferred PCBs detected in northern pikeminnow ovary/oocyte samples were not alarming. A total of seven different PCB congeners were detected in one or more of the samples (Table 3). PCB 153 was detected the most frequently (6/10 samples) and at the greatest concentration, (up to 11.2 ng/g; Table 3). PCBs 110, 118, and 138 were each detected in a single Newberg fish.

All PCB congeners detected were mono- or diortho-substituted. These congeners tend to be much less toxic than nonortho planar PCBs (*59*, *62*, *63*). Fish, in particular, have been shown to be less sensitive to the mono-ortho PCBs than mammals or birds (*63*−*64*). It has even been suggested that coexposure to the relatively nontoxic mono- and diortho PCB congeners may reduce the overall uptake of the more toxic nonortho planar PCBs (*59*). Mean concentrations of PCBs 8 and 18 were greater in fish collected from Corvallis than from Newberg (Table 3). Conversely, the mean concentration of PCB 153 in ovary/oocyte tissue was greater in Newberg fish (Table 3). However, neither of these differences was significant. On the basis of the samples analyzed, maternal transfer of PCBs does not appear to pose a high risk of overt early life stage toxicity to Willamette River northern pikeminnow.

PCDDs and PCDFs were detectable in all ovary/oocyte samples. Specific congeners varied considerably among samples; therefore, a toxic equivalents approach (*64*) was used to facilitate analysis of the results and comparison among sites. Total 2,3,7,8-TCDD equivalents in oocyte/ovary tissue of Willamette River northern pikeminnow ranged from 0.18 to 2.06 pg/g wet wt. The greatest TEQ concentration (2.06 pg/g wet wt) was detected in a fish from Corvallis; however, the mean TEQ concentrations were nearly identical for fish collected from the two study sites (0.84 ± 0.20 versus 0.85±0.31 pg/g wet wt for Newberg and Corvallis, respectively; Table 3). Total concentrations of TEQs were less than those expected to cause toxicity during early life stage development. On the basis of measured TCDD concentrations in fish eggs, the lowest observed effect concentration for seven different fish species ranged from 270 to 2000 pg/g wet wt (*65*). NOECs were > 175 pg/g wet wt (*65*). The TEQ concentrations detected in this study were at least 135 times lower than the LOEC for the most sensitive of the seven species tested (*65*). Furthermore, the TEQ concentrations detected were at least 2.5 times less than the probable no-observable-adverse-effect level (NOAEL) of TEQs for lake trout, which is widely regarded as the fish species most sensitive to dioxin and dioxin-like toxicity (*65*, *66*). Thus, concentrations of maternally transferred PCDDs and PCDFs did not appear likely to cause early life stage mortality.

On the basis of the literature, it was unclear whether any of the concentrations of POPs detected in Willamette River northern pikeminnow ovary/oocyte tissue would be likely to cause skeletal deformities. However, no significant differences in maternally transferred POP concentrations were observed for fish from the Newberg versus Corvallis sites (Table 3). Even if early life stage exposure to POPs was causing some disruption of early development, leading to skeletal deformities, it was unlikely to account for 2−3-fold greater rates of skeletal deformities. As a whole, these results provided no compelling evidence to support the hypothesis that greater maternal transfer of POPs was a likely cause for the greater incidence of skeletal deformities in fish from the Newberg region of the Willamette River.

Sediment POPs. A small number ($n = 3$ per site, per year) of surficial sediment samples collected from Newberg and Corvallis sites were analyzed for persistent chlorinated pesticide residues and PCBs to determine whether trophic transfer of these compounds from sediment, or direct exposure of embryo-larval fish (particularly for broadcast spawners; (*40*)) could account for differences in deformities at the two sites. Chlorinated pesticides were not detected in samples collected from Corvallis or Newberg in either 2002 or 2003. In 2002, PCB 8 was detected in 2/3 Corvallis samples and 1/3 Newberg samples. Concentrations of PCB 8 ranged from 1.3 to 6.6 ng/g. Additionally, PCB 128 was detected in a single Corvallis sample and PCBs 18, 101, and 153 were detected in a single Newberg sample. Concentrations of these congeners ranged from 0.5 (PCB 101) to 3.8 (PCB 18). In 2003, only 2 congeners, PCB 101 and PCB 110, were detected. PCB 101 was found in one Corvallis sample and two Newberg samples at concentrations ranging from 0.37 to 1.2 ng/g. PCB 110 (1.1 ng/g) was detected in a single Corvallis sample. Samples were not collected at identical locations each year, so it was not possible to determine whether differences in the congeners detected were the result of spatial or temporal differences. Overall, the results did not provide compelling support for the hypothesis that chlorinated pesticide residues or PCBs present in surficial sediments were a likely cause for the greater skeletal deformity load in Newberg fish.

Skeletal Deformities Bioassay I: River Water Extracts. Results of laboratory exposure of fathead minnows to Willamette River water extracts from d 2 to d 6 post-hatch with subsequent grow-out to d 28−30 post-hatch did not provide evidence that unknown compounds or interactions between chemicals present in the prepared extracts were likely causes of greater deformity loads observed in fish from certain regions of the Willamette River. Survival to d 6 post-hatch ranged from 83 to 100% in all trials, and there were no significant differences among treatments ($p = 0.202-0.754$), indicating that extracts were not acutely toxic to larval fathead minnows. Survival during grow-out was variable among replicates and among trials, ranging from 5 to 19 fish per replicate (17−63%). In all cases, a minimum of 20 fish per treatment group were examined for deformities. It was not possible to determine whether fish that died during grow-out were deformed. Nonetheless, the lack of a significant treatment-related effect on survival to the examination day ($p = 0.425-0.980$) suggests that the mortality during grow-out was randomly distributed among replicates and did not obscure a treatment effect.

When simple dorsal−ventral curvature was included as a deformity, 5−25% of the fish examined were classified as deformed, although no treatment-dependent effect was observed ($p = 0.834-0.929$). When the analysis was restricted to only those deformities characterized as "qualitatively similar to those observed in Willamette River fish" (as per categories defined by Cunningham et al. 2004 (*40*)), the incidence of deformities ranged from 0.8%-2% for the entire population surveyed in each trial. Given total sample sizes of 210−397 fish per trial, this represented 2−8 fish. In all cases, deformities were spread across treatments, such that no association with any particular treatment was evident.

The distribution of developmental scores was unaffected by treatment in the NP/CV and AI/CV trials ($p = 0.255, 0.470$), with most fish having developmental scores greater than 3. In the WF/CV trial, fish from the 4XC group were significantly more developed than those from all other treatment groups ($p = 0.024$). However, no concentration-dependence was evident. As a whole, there was no evidence that Willamette River water extracts induced skeletal deformities or otherwise adversely affected larval fathead minnows exposed for 96 h from d 2 to d 6 post-hatch.

It must be noted, however, that a negative response in the skeletal deformities bioassay did not rule out the possibility that chemicals contained in the extracts had the potential to induce deformities in cyprinid fish. As designed, the assay was able to provide a reasonable screen for the potential of the river water extract to disrupt some early life-stage developmental processes important for formation of the ossified vertebral column. The assay was not expected to be an effective screen for chemicals able to cause skeletal deformities through acute neuromuscular damage. A time-series for skeletal development in FHM held under assay conditions showed that as early as d 5 post-hatch, nearly all fish had ossified skulls and partial vertebral formation, as indicated by ossification of the anterior-most centra (unpublished results). Attempts to validate the assay using Cd,

Se, and chlorpyrifos as positive controls were unsuccessful, as deformities were not induced at nontoxic concentrations (unpublished results). Robust application of the method will require additional characterization of the detectable mechanisms of action, and further optimization to reduce mortality-related variability during grow-out.

Association of Parasites with Deformities in Field Collected Fish. The occurrence of skeletal deformities in Willamette River fish was strongly linked with metacercariae of a digenean trematode, likely *Apophallus donicus* (*40*, *47*, *48*). An analysis of cleared and stained specimens of northern pikeminnow and chiselmouth collected from four Willamette River locations, including Newberg, Wheatland Ferry, and Corvallis (Figure 1), concluded that the probability of having a precaudal skeletal deformity was strongly dependent on the number of trematode cysts in the body ($p < 0.0001$) and the location in the river ($p = 0.006$) (*40*). Species and fish size were not significant predictors (*40*). Trematodes were directly associated with 86.5% of 592 primary precaudal deformities detected in chiselmouth and 46.3% in northern pikeminnow (*40*, *47*). Additionally, a *Myxobolus* sp., likely *Myxobolus cyprini*, was associated with a significant percent (36%) of northern pikeminnow with histologically verifiable skeletal deformities (*47*). These results suggested that parasites were a likely cause for the skeletal deformities observed in Willamette River fish. However, solely on the basis of examination of field collected specimens, it was not possible to determine whether parasites were actually causing deformities or whether deformed fish were simply more vulnerable to infection.

Skeletal Deformities Bioassay II. Exposure to *Apophallus donicus* Cercariae. Results of the laboratory infection studies convincingly demonstrated that vertebral deformities consistent with those observed in Willamette River fish could be caused by trematode cercariae identified as *Apophallus donicus* (Figure 2). Five separate exposure trials were conducted with fathead minnows (a cyprinid species) ranging from 8 to 24 days old (post-hatch). Mortality was variable and often high in both cercariae-exposed (14−71%) and control fish (5−91%). Nonetheless, conclusions could be drawn. A high incidence of infection (80−100%) was observed in cercariae-exposed fish from all trials, and infected fish exhibited a high incidence of vertebral deformities (70−93%; Table 4). Most deformities were directly associated with metacercariae (Figure 2), and nearly all trematodes were directly located along the vertebral column. The types of deformities observed were also identical to those observed in field-collected specimens (*40*, *47*) including extra spines, lordosis, fused vertebrae, and increased vertebral density (Figure 2). As in field-collected specimens, metacercariae occurred directly appressed to or deep within vertebrae and were often associated with bone hypertrophy. In contrast to cercariae-exposed fish, only 4% of the control fish examined exhibited skeletal deformities (Table 4). Control deformities were characterized as curvature of the spine or fused vertebrae. The incidence of skeletal deformities in control fish was similar to background rates of skeletal deformities determined for lab-reared fathead minnows examined by fluorescence microscopy as part of exposures to river water extracts.

Controlled laboratory exposure to *A. donicus* replicated vertebral deformities observed in fish collected from the field and further demonstrated that this parasite was likely a major cause of deformities in Willamette River cyprinid fish. This heterophyid digenean trematode exhibits broad host specificity, infecting many species in the family Cyprinidae as well as fish from several other families (*48*). As observed in both our laboratory and field studies, the parasite exhibits remarkable affinity for bone (*40*, *47*). Most of the metacercariae were associated directly with skeletal structures and were not found in the viscera. Similar to *Apophallus* sp. in the present study, *A. brevis* in yellow perch apparently does not infect the visceral organs (*67*). Taylor et al. (*68*) described bony ossicles in yellow perch caused by *A. brevis*. Infections by other metacercariae types have been linked to vertebral anomalies. Muscle infections by *Bucephalus polymorphus* caused vertebral deformities in cyprinid fishes (*69*), and *Riberiora sp.* was suspected to be a major cause of supernumerary limbs and other vertebral changes seen in North American frogs (*70*). Thus, both empirical evidence and literature reports support the conclusion that trematode parasites are causing skeletal deformities in Willamette River fish.

Future Investigation. Although parasitic infection is likely the primary cause of skeletal deformities in Willamette River fishes, questions remain as to whether spatial differences are due to natural or anthropogenic factors. Increased occurrence of trematode infections have been linked to anthropogenic pollution and physical alteration of aquatic habitats caused by human activities (*71*). Potential synergism between exposure to herbicides and pesticides and susceptibility of frogs to infection by metacercariae of *Ribeiroia sp.* and *Telochris sp.* have also been reported (*72*). None of the chemical contaminants detected in this study are known to cause immune suppression or increase susceptibility to infection at the concentrations observed. However, the biological assays used in this study did not test the interaction between exposure to parasites and exposure to complex mixtures of chemicals present in Willamette River water or sediment extracts. This would be a useful step toward determining whether these chemicals promote susceptibility to parasites. Alternatively, it is possible that spatial differences in deformities reflect a natural phenomenon. Given the life cycle of *Apophallus donicus* (*48*), any habitat characteristics favoring either the intermediate host (snails such as *Fluminciola virens*) or the definitive host (fish-eating birds) could result in greater *Apophallus sp.* abundance and potentially more infections. Natural factors, influencing the viability and numbers of microbial and other infectious agents, such as ORP, may also play a role. Additional study parasite ecology and potential interactions with anthropogenic influences could help determine appropriate management actions for affected regions of the Willamette River basin.

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Supporting Information Available

(1) List of all target analytes and parameters analyzed as part of in situ monitoring with a YSI 6920 Sonde probe and in situ sampling of bioavailable organic compounds and metals using PSDs and DGTs, (2) diagram of float-cable-cage-cable-anchor setup, (3) 2002 and 2003 pH and ORP trends, (4) list of target analytes analyzed in ovary/oocyte tissues and surficial sediments and the concentrations detected in each sample, (5) developmental scoring criteria used for fathead minnow skeletal deformities assay, and (6) examples of deformities observed in fathead minnow skeletal deformities assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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