DELAYED EFFECTS OF OIL EXPOSURE ON FISH

By
Corinne E. Hicken

RECOMMENDED

Dr. Stanley D. Rice

Dr. William W. Smoker

Dr. John P. Incardona

Dr. Michael S. Stekoll, Advisory Committee Chair

Dr. Milo Adkison
Chair, Graduate Program in Fisheries Division

APPROVED

Dr. Michael Castellini
Dean, School of Fisheries and Ocean Sciences

Dr. Lawrence K. Duffy, Dean of the Graduate School

April 11, 2012
Date

DELAYED EFFECTS OF OIL EXPOSURE ON FISH

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

of the Degree of

MASTER OF SCIENCE

By

Corinne E. Hicken, B.S.

Fairbanks, AK

May 2012

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are continuously added to aqueous environments through point source and non-point source pollution and can cause deleterious effects on exposed fish populations. Historically, studies have shown that acute PAH exposure causes only short-term effects in adult fish which were resolved when the exposure ended. Chronic exposure to PAHs, however – even at the less susceptible juvenile and adult stages – can cause a host of effects including lesions, lower body length and weight, and reduced swimming ability. More recently studies of embryonic fish have demonstrated that much lower PAH concentrations can cause lethal and sub-lethal effects on those embryos and can cause delayed effects on the fish that are not seen until adulthood. This study used zebrafish (*Danio* rerio) to examine the effects of 48-hour weathered crude oil exposure on both the embryonic fish exposed and the adult fish exposed as embryos but raised in clean water. Oil exposed embryos had increased mortality, pericardial edema, intracranial hemorrhage, and higher cytochrome P4501A (CYP1A) activity. Adult fish exposed as embryos had decreased critical swim speed and rounder hearts than the control fish. These effects may culminate in decreased fitness of the exposed fish population.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Michael Stekoll for his guidance and support. Thank you to my committee Dr. Stanley Rice, Dr. John Incardona and Dr. William Smoker for all of their help. Thank you to Tiffany Linbo at the Northwest Fisheries Science Center for all of her fish husbandry support. Thank you to Dr. David Baldwin at the Northwest Fisheries Science Center for statistical analysis advice. This publication is the result of research sponsored by Alaska Sea Grant with funds from the National Oceanic and Atmospheric Administration Office of Sea Grant, Department of Commerce, under grant no. NA06OAR4170013 (project no. R/97-01), and from the University of Alaska with funds appropriated by the state. Some of the results of this research have been published:

Hicken, C.E. T.L. Linbo, D.H. Baldwin, M.L. Willis, M.S. Meyers, L. Holland, M. Larsen, M.S. Stekoll, S.D. Rice, T.K. Collier, N.L. Scholz, and J.P. Incardona. 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Nat. Acad. Sci. 108(17): 7086-7090.

Introduction

Contaminants such as polycyclic aromatic hydrocarbons (PAHs) from fossil fuels are constantly added to our aquatic environments, not only through point-source pollution (e.g. the 1989 *Exxon Valdez* oil spill), but more insidiously through non point-source pollution. Non-point source pollution (e.g. urban runoff and automobile exhaust) near urban areas throughout the United States leads to chronic exposure situations with concomitant problems. Growing urbanization in coastal areas is likely to increase the potential for chronic exposure to PAHs of resident fish populations. Of especial concern are those species with sensitive life stages that rear in urban estuaries. Fish populations in places like Chesapeake Bay, the Great Lakes, Puget Sound, and New York Bight are rarely exposed to large-scale events such as the *Exxon Valdez* oil spill, but are commonly exposed to small-scale events whose additive effects are likely to be even more debilitating than those caused by acute exposures (Marty *et al.* 1997; Carls *et al.* 1999; Heintz *et al.* 2000).

Previous studies of the effect of PAH or crude oil exposure on fish focused primarily on post-embryonic life stages. Short-term exposure (48-96 hours) studies were often determined to have little to no effect on fish. Coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*O. nerka*) parr exposed for 96 hours to crude oil poured on the water's surface had higher mortality than control fish, but this difference disappeared when the oil was exposed to air before dosing the fish (Morrow 1973). That weathering the oil eliminated the mortality difference indicated the mortalities were caused by narcosis. Crude oil was also shown to accumulate in juvenile coho salmon exposed for 96 hours, but the fish were able to rid their tissues of any accumulated oil in three weeks (McKeown 1981). These and similar studies suggested that any effects of oil exposure were short-term and impermanent.

Experiments using multi-day or chronic crude oil doses have produced different results. Because post-embryonic life stages are less sensitive to PAH injury, higher chronic doses have been used to discover an effect. Growth of cutthroat trout (*O. clarki,* as *Salmo*

clarki) was reduced in alevins exposed for 60-90 days to 100-520 µg/L of Wyoming crude oil (Woodward *et al.* 1981). Caudal fin erosion was seen in the 150-520 µg/L doses after 90 days. Gill and eye lesions also developed at the highest dose levels after 90 days (Woodward *et al.* 1981). Liver, head, kidney and gill lesions also developed in pink salmon (*O. gorbusha*) fry after a 10-day exposure to 25-54 or 178-348 µg/L crude oil (Brand *et al.* 2001). These lesions can be detrimental to the performance of the organs or structures affected. Juvenile rainbow trout (*O. mykiss*), after a 21-day pre-exposure to 50 and 100 mg/L of No. 2 fuel oil, had decreased survival when subsequently dosed with an acute lethal level of the fuel oil when compared to unexposed fish (Steadman *et al.* 1991). A 25-day exposure to heavy fuel oil caused a decrease in heart contraction rates, respiration frequency, body weight and length, and swimming ability in rainbow trout alevins (Stasiunaite 2003). These later life stages require relatively high, long-term exposure to cause observable damage.

However, embryonic fish can be affected by much lower and shorter-term PAH or crude oil exposure. Exposure to low levels of PAHs is often lethal in early life-stage fish. For example, exposure to weathered Alaska North Slope (ANS) crude oil caused increased mortality in Pacific herring (*Clupea pallasi*) and pink salmon embryos and larvae (Carls *et al.* 1999; Heintz *et al.* 1999). Complete mortality was seen in zebrafish embryos exposed to dibenzothiophene, pyrene, and phenanthrene (Incardona *et al.* 2004). This lethality can also be enhanced by phototoxicity in translucent eggs and larvae (Barron *et al.* 2003).

Further, PAHs in lower concentrations can also cause a suite of sub-lethal effects in early life-stage fish. Embryonic PAH exposure causes a large number of sub-lethal but crippling syndromes, including ascites, pericardial and yolk-sac edema, developmental defects (including craniofacial malformations, skeletal defects, and abnormal fin development), premature emergence, delayed or failed swim bladder inflation, and cardiac function defects (Marty *et al.* 1997; Carls *et al.* 1999; Heintz *et al.* 1999;

Incardona *et al*. 2004). All of these effects have been seen in embryonic fish during or immediately after PAH exposure.

Sub-lethal effects like those listed above are likely to be subtle, but can ultimately lead to many ecosystem changes. Interactions among species in an ecosystem may be altered by sub-lethal effects of contaminants. For example, reductions in salmon escapements can lead to reduced inputs of marine-derived nutrients to riparian habitats (Wipfli *et al.* 1998), which may significantly reduce productivity of the salmon populations that spawn in those habitats (Cederholm *et al.* 1999; Gresh *et al.* 2000; Wipfli *et al.* 2003; Wipfli *et al.* 2010). Contaminated waters have led to a decreased predatory ability of the fish *Fundulus heteroclitus*, eventually leading to an increase in abundance of their prey (Weis *et al.* 2001). But prey species can also be negatively affected as well. The clam *Macoma balthica*, when exposed to crude oil in seawater, exhibited decreased burrowing and growth rates and reabsorbed their gametes (Stekoll *et al.* 1980). Any population decreases caused by such sub-lethal changes could affect the populations of shore birds that prey on *M. balthica*. The black oystercatcher (*Haematopus bachmani*) avoided oiled areas in Prince William Sound after the 1989 Exxon *Valdez* oil spill (Sharp *et al.* 1996). This was likely in response to the mussels on which they prey entering an inactive, closed state after the oil spill (Sharp *et al.* 1996).

Sub-lethal injury caused by PAH exposure may not produce effects until later in life, after exposure has ceased. Delayed effects such as these are an important, but poorly understood, group of exposure effects. Such effects can appear long after exposures to PAHs, during early developmental stages, have ended. Injuries acquired at this stage may not be immediately lethal; consequently they are not detected by traditional short-term bioassays. However, these sub-lethal injuries may interfere with vital functions during later life stages. For example, embryonic exposure to crude oil has been shown to lead to reduced growth, survival (Heintz *et al.* 2000; Carls, *et al.* 2005), and possibly reproductive output (Bue *et al.* 1998) in pink salmon later in development. Wertheimer *et al.* (2000) found that pink salmon exposed to oiled gravel had increased straying rates

compared to control fish, though their results were not significant. Lower growth rates in pink salmon, while not immediately lethal, may cause reduced marine survival, possibly through size-selective predation (Willette *et al.* 1999; Moss *et al.* 2005).

Embryonic PAH exposure can also cause delayed effects on the cardiac function of adult fish. Functional valve formation, a final milestone in zebrafish cardiac morphological development, is complete at 48 hours post fertilization (hpf), and the development of the heart morphology and function are closely linked (Glickman and Yelon 2002). Embryonic fish hearts can fail to develop, becoming atretic (string-like) when constantly exposed to toxins such as PAHs, a condition which is ultimately fatal to the embryo. Sub-lethal exposure to PAHs could cause a slight malformation in a developing fish heart, leading to reduced fitness in adulthood. Ventricle shape has been shown to affect swim performance and cardiac output in fish. Hatchery-raised rainbow trout (*Oncorhynchus mykiss*) with relatively smaller ventricles were poorer swimmers (Graham and Farrell 1992). Poor swimming ability has been linked to reduced cardiac output in fish (Claireaux *et al.* 2005). Fast-swimming fish like salmonids and clupeids have pyramid-shaped ventricles, which have higher cardiac outputs (Agnisola and Tota 1994; Sanchez-Quintana *et al.* 1995). Fish with pyramid-shaped ventricles have had lower swim performance when those ventricles are more circular (smaller length-width ratios) (Claireaux *et al.* 2005).

Evaluations of the toxicity of contaminants that cause delayed effects generally result in identification of toxicity levels much lower than those determined by short-term bioassays, due to detailed accounting of the delayed impacts on population survivorship and reproductive output after exposure of the most sensitive life stages. For example, Heintz *et al.* (2000) determined that exposure of pink salmon embryos to concentrations of PAHs in the low parts-per-billion led to lower marine survival in adult pink salmon. White *et al.* (1999) found reduced hatch frequency and larval survival in adult fathead minnows whose parents were exposed to similar parts-per-billion concentrations of benzo-[a]-pyrene as larvae. Smoker *et al.* (2000) saw reduced fertility in the unexposed

offspring (F1) of pink salmon who had been exposed to crude oil. In addition they found early hatching and increased phenotype variation in that F2 generation. If all of these delayed effects were combined in a single population, then average fitness would be significantly reduced, casting doubt on the long-term viability of the population (Heintz 2007).

The much lower concentrations of a contaminant that cause sub-lethal or delayed effects should be considered when setting water quality standards and water quality criteria. Historically, water quality criteria for oil exposure nationwide have been based on LC50s calculated from 96-hour acute toxicity studies (Quality Criteria for Water 1986). These tests do not simulate realistic exposure conditions, where multiple life stages are exposed to low levels of toxicants, and there are few studies of the delayed effects of short-term exposure. The studies mentioned above suggest that the existing water quality criteria may underestimate the ultimate effect of toxicants on exposed populations. This situation may lead to the establishment of standards that are too high relative to the true concentrations that will cause detrimental effects.

In order to address the question of delayed effects of PAH exposure to fish, we developed an hypothesis predicting that early developmental stages of organisms will suffer sub-lethal injuries as a result of their exposure to low levels of PAH contaminants, and that the effects of those injuries are manifested over the lives of the animals. These effects may include development of liver neoplasms (Baumann and Harshbarger 1995), increased susceptibility to predation (Kruzynski and Birtwell 1994), reduced growth rates and marine survival (Heintz *et al.* 2000), impaired reproductive ability (White *et al.* 1999), reduced fish mass (Carls *et al.* 2005), and decreased cardiac function (Incardona, *et al.* 2004). Those effects are consequences of the exposures and are likely to be expressed regardless of whether the exposure continues though adulthood.

One method to assess sub-lethal effects of pollutants on fish is to measure swim performance, a technique that has long been used in fish physiological studies (Beamish

1978). Methods of measuring swim performance vary widely, using a range of different apparatus and procedures (Beamish 1978). A commonly used method employs a swim chamber to expose fish to incrementally increasing water flow velocities until the fish fatigue (Beamish 1978). Fatigue is typically defined as when the fish is unable to continue to swim against a current. The time to fatigue is then used to calculate critical swim speed (U_{crit}), a reliable measure of maximum aerobic performance. U_{crit} is a calculation of the maximum velocity a fish can maintain for a designated time period (Brett 1964). Claireaux *et al.* (2005) found that rainbow trout (*Oncorhynchus mykiss*) with increased cardiac pumping ability had a higher U_{crit}.

In this study we used zebrafish (*Danio rerio*) to model delayed effects to fish of embryonic exposure to weathered Alaska North Slope (ANS) crude oil. Zebrafish are an ideal model for several reasons. They are oviparous with a short generation time and can be reared easily in a laboratory setting. They have been used extensively as models in toxicology studies (Samson *et al.* 2001; Smolders *et al.* 2002) and their genetics are well understood. Zebrafish have pyramid-shaped heart ventricles which can be examined histologically to identify morphological differences. Zebrafish eggs and larvae have been shown to bioaccumulate PAHs and show toxic responses to those compounds (Petersen and Kristensen 1998). Incardona *et al.* (2004) found that zebrafish embryos exposed to several 2-4 ring PAH compounds developed a suite of defects, including dorsal curvature of the tail and trunk, pericardial and yolk-sac edema, reduced growth of the head, and cardiac function disruption.

PAH exposure, to either single compounds or to weathered crude oil, has been shown to differentially induce cytochrome P4501A (CYP1A) expression in zebrafish (Incardona *et al.* 2005, Incardona *et al.* 2006). CYP1A induction has long been used as a bioindicator of PAH exposure in fish (Spies *et al.* 1996; Marty *et al.* 1997; Stagg *et al.* 2000; Incardona *et al.* 2006; Wills *et al.* 2009). CYP1A aides in PAH metabolism by increasing their solubility, making them easier for organisms to excrete. This process, however, can generate toxic or mutagenic metabolic intermediates. Benzo[a]pyrene, for

example, can be metabolized into many reactive compounds including BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), which is highly mutagenic (Ericson and Balk 2000). However, Incardona *et al.* (2005) also demonstrated that PAH toxicity can be activated through metabolic pathways independent of CYP1A induction.

Our objectives were to develop a model laboratory system for evaluating the delayed effects of organic pollutants on exposed fish populations, and to use that system to examine the delayed, sub-lethal effects of crude oil exposure on the survival and physiological fitness of exposed fish. We exposed 4-8 hpf zebrafish embryos to water that had been in contact with variously-aged oil coated gravel, and then reared the fish to adulthood. Aside from mortalities, we monitored developmental abnormalities, CYP1A induction, and measured the swim capacity of adults. We also examined heart morphologies histologically.

Materials and Methods

Zebrafish culture and exposures

All rearing, experimental oil exposures and physiological experiments were conducted at NOAA's Northwest Fisheries Science Center in Seattle, WA under University of Alaska IACUC assurances 02-28 and 05-52. Zebrafish (*Danio rerio*) wild-type AB broodstock (originally from the University of Oregon Zebrafish Facility, http://fish.uoregon.edu/zf/index.html) were maintained in tanks kept on a 14 hour light/10 hour dark cycle at 28.5° C. Methods described in Westerfield (1995) were used to maintain the adult zebrafish. Fish spawning, exposures, and adult zebrafish were all maintained in "system water", defined as reverse-osmosis water with Instant Ocean® sea salt added to adjust it to a conductivity of approximately 1500 µS/cm and a pH between 7-8. The procedure for spawning involved placing three females and two males together in a tank with a perforated bottom the afternoon before eggs were needed. Zebrafish generally spawn within four hours of "dawn". Fertilized eggs were collected within two

hours of spawning. Eggs used in the exposures were all between the four- and eight-cell stage at the start of the exposure.

We used oiled gravel columns as previously described in Incardona *et al.* 2005 to expose the zebrafish embryos to partially weathered Alaska North Slope (ANS) crude oil. Gravel 4-6 cm in diameter was coated with the crude oil (6 g oil/kg gravel). 1.3 kg of oiled gravel was placed in a 2 L glass beaker. A similar column which contained clean (unoiled) gravel was employed for control fish incubation. System water was directed to the bottom of the beakers through 6 mm glass tubes using centrifugal pumps to maintain a constant flow of 10 mL/min. The resulting effluent overflowed the beaker tops and was collected in rectangular 23 x 33 cm glass baking dishes, set at a slight angle to allow the water to flow out the far end. Embryos were placed in open 60 mm x 15 mm diameter glass Petri dish replicates (n=5, 30 embryos per dish) set in the baking dishes. The temperature in each baking dish was maintained with submersible heaters, and recorded up to three times daily. Dishes were maintained at temperatures between 27° and 28.5° C. All exposures were 48-hours long.

System water was pumped continuously through both the oiled and control gravel columns 24 hours a day, seven days a week, for two months. During this time, new embryos were exposed on average every three to four days (Table 1). Each batch of embryos is identified by the age of the column when its exposure began. For example, the first batch of embryos was added to the exposure system on Day 2 (48 hours after water flow through the gravel began), the second on Day 4, etc. After each 48-hour exposure, embryos were subsampled and preserved in paraformaldehyde for CYP1A analysis. At this point the remaining embryos were anesthetized and discarded, except for those from days 23 (Clutch 1), 33 (Clutch 2) and 42 (Clutch 3) which were transferred to clean water and placed in incubators at 28.5° C. These clutches contained embryos with survival potential, unlike the earlier exposures that contained few if any potential survivors. The embryos remained in the incubators for an additional 48 hours, at which point they were transferred to a temperature-controlled zebrafish facility and reared to

adulthood. Mortality was assessed daily for 12 days post fertilization (dpf) while the embryos were separated from the adult zebrafish colony.

Table 1. Start dates of 48-hour zebrafish embryo clutch exposures to control and oiled gravel columns over the course of the experiment. Clutches marked with an asterisk (*) were raised to adulthood.

Exposure Start Date	Age of Column (days)
6/22/2007	2
6/24/2007	4
6/26/2007	6
6/28/2007	8
6/30/2007	10
7/2/2007	12
7/4/2007	14
7/10/2007	20
7/13/2007	23*
7/16/2007	26
7/19/2007	29
7/23/2007	33*
8/1/2007	42*

Pericardial Edema and Intracranial (I.C.) Hemorrhage

A stereo microscope was used to determine the presence or absence of pericardial edema and hemorrhaging in a sample of 30 fish. Each embryo was examined, and if any fluid was collected around the heart (pericardial edema) or if any bleeding was seen in the head (i.e. hemorrhage) than the embryo was recorded as having the condition.

CYP1A Immunofluorescence and Confocal Microscopy

CPY1A immunofluorescence processing was carried out as previously described (Incardona *et al.* 2004 and Incardona *et al.* 2005). In general, embryos were fixed overnight in 4% phosphate-buffered paraformaldehyde (pH=7), and then transferred to methanol plus 10% DMSO for storage. Primary monoclonal antibodies were used to

bind directly to the CYP1A antigens. Secondary antibodies with fluorochrome labels bound to the primary antibodies for fluorescent detection. Primary antibodies used were monoclonal 1-12-3 against fish CYP1A (Park *et al.* 1986), anti-myosin heavy chain monoclonal MF20 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) (Bader *et al.* 1982), and anti-atrial myosin heavy chain monoclonal S46 (Berdougo *et al.* 2003). Secondary antibodies used were AlexaFluro488-conjugated goat anti mouse IgG1 (S46) and AlexaFluro568-conjugated goat anti-mouse IgG2b (MF20), both from Molecular Probes (Eugene, OR, USA). Mounted immunolabeled embryos were imaged using a Zeiss LSM 5 Pascal confocal system with AR and He/Ne lasers (Carl Zeiss Advanced Imaging Microscopy, Jena, Germany). All CYP1A staining and confocal microscopy was performed by John Incardona at NOAA Northwest Fisheries Science Center in Seattle, WA.

Histology

Live adult zebrafish selected for assessment of histopathological changes were euthanized and any external abnormalities (e.g., frayed fins, cloudy eyes, ulcers, skin discolorations, parasites) were assessed visually. The gills were similarly examined after cutting away the operculum. Internal organs were also examined after making a midline incision on the belly from the anus to the pectoral girdle, and assessing for ascites, hemorrhage or other abnormalities. The visceral cavity was then further exposed by removing a small section of the abdominal wall, and the tail was excised posterior to the anus. Fish for histology were preserved whole in Dietrich's fixative solutions for histology, at approximately 1:20 (v/v) tissue to fixative, typically requiring 15 mL fixative for 1-2 zebrafish. To ensure uniform and complete fixation, tissues were fixed for three days on a rotor or other agitating device. The entire fish could then be processed (usually cut in sagittal sections) and representative organs visualized in multiple sections on one or two histological slides.

After three days, the samples were removed from fixative, rinsed in two or three changes of water, and placed in 70% ethanol. The fish were then bisected along their length using

a fresh razor blade, making the cut parallel to and on the left side of the spinal cord, before further processing. The bisected fish were then loaded into cassettes using a Shandon Hypercenter XP tissue processor (Thermo Scientific, Waltham, MA). The tissue processing protocol followed that specified by the Zebrafish International Research Center, University of Oregon, Eugene, OR. Processed tissues were infiltrated with a 50:50 ratio of Fisher Paraplast PLUS tissue embedding medium (Fisher Scientific, Pittsburgh, PA) and Surgipath Formula 'R' infiltrating and embedding paraffin (Surgipath Medical Industries, Richmond, IL). Tissues were then embedded in Surgipath Formula 'R' paraffin.

Tissues were cut, either in step or serial sections, as needed, at 5-7 μm thickness with a high-profile disposable blade. If a fish was difficult to cut, the block face was soaked in ice-cold water with 5 ml Tween 20. Because this study focused on the morphology of the heart, multiple serial sections of each fish were cut until it was possible to view a full section of the ventricle, atrium, and bulbus arteriosus, with the key feature being a full longitudinal section of the bulbus arteriosus. This was done to standardize the orientation and sectioning plane of the three heart chambers as much as possible for imaging and measurements.

Sections of gills, spinal cord and all internal organs were taken to ensure proper pathological evaluation. Because the fish had been cut to one side of the spinal cord, sections were cut toward the midline on one half of the fish, and away from the midline on the other. This produced a ribbon of sections containing gills (one bisected half) and spinal cord (the other bisected half), along with a complete sampling of internal organs, while continuing to focus on the plane of section of the heart components.

Sections were routinely stained by hematoxylin and eosin, using the protocols as described in Stehr *et al.* (1993). Sections were screened by light microscopy for proper heart visualization and orientation and for digital photomicroscopy. Sections were examined and imaged with a Nikon E600 compound microscope. All histology

sectioning and analysis was performed by Maryjean Willis, Mark Myers, and John Incardona at NOAA Northwest Fisheries Science Center in Seattle, WA.

Swim Performance Measures

Swim performance of control and exposed zebrafish was determined with a rectangle-shaped swim tunnel, using a design modified from Ward *et al.* (2002) (Fig. 1). Water current was generated by a magnetic drive pump, and the flow was controlled by a knife gate valve located directly downstream from the pump. The fish testing chamber was located in a section of clear PVC, in order for the researcher to observe and measure fatigue. Other knife gates, screened and solid, prevented the fish from moving out of the testing chamber. Straws and varying diameters of pipe helped to linearize the flow through the testing chamber. A flow meter set up in the swim tunnel measured flow in liters per minute. An electric barrier was installed immediately downstream from the testing chamber, to discourage fish from resting against the downstream screen.

Five fish (ages 10-11 months) were used in each swim trial. They were placed in the swim tunnel and allowed to swim to the testing chamber. Once in the testing chamber, the screened knife gate was lowered to prevent escape. The fish were allowed to acclimate in the testing chamber for 10 minutes before a low flow (6.2 cm/s) was introduced. The flow was maintained at 6.2 cm/s for 30 minutes, to allow the fish to orient themselves to the flow direction. After 30 minutes, the flow was increased to 10.3 cm/s and maintained there for 20 minutes. Then the flow was increased in 10.3 cm/s increments every 10 minutes until the first fish was fatigued. Fatigue was defined as when a fish fell against the downstream screen and could not recover despite prodding from the electric barrier. Once fatigue was determined, the trial was ended and the fish were removed from the swim tunnel. All fish were euthanized after each trial, and their lengths and weights were recorded.

Condition factor was calculated using this formula (Williams 2000):

$$K = \frac{100{,}000W}{L^3}$$

Where: W = weight of the fish in grams;

L = length of the fish in millimeters.

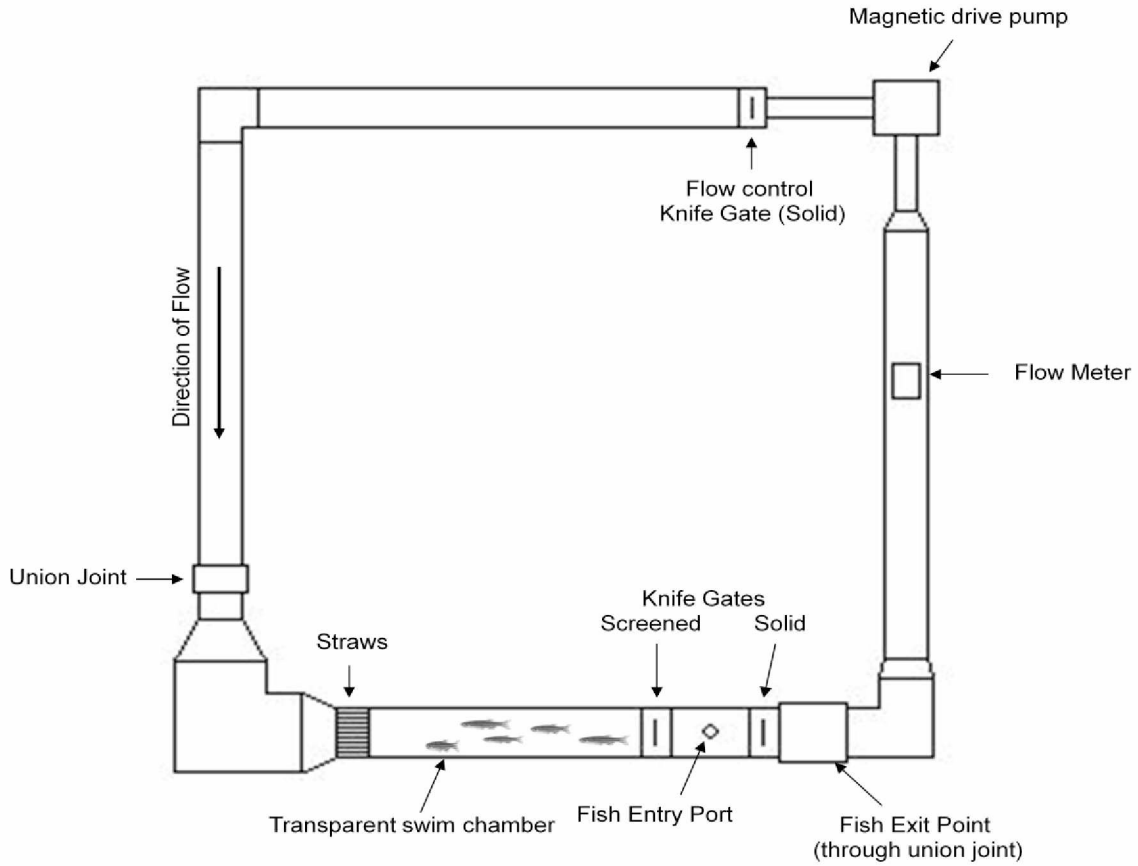

Figure 1. Diagram of the swim tunnel used in swim performance trials.

PAH analysis

One water sample (200 mL) was taken at the beginning and end of each exposure from both the control and oiled gravel column effluents. The samples were stored and extracted as previously described (Incardona, et al. 2005). Each sample was stored in a brown glass bottle with the addition of 20 mL dichloromethane in a 4° C refrigerator until extraction. After deuterated internal standards were added, each sample was extracted twice with 25 mL dichloromethane. The extractions were stored in a -20° C freezer until further processing to remove any residual water. The solvent dichloromethane was replaced with hexane via boiling evaporation as outlined in Larsen *et al.* (2003). Boiling stones were added to each sample extract, and the extracts were placed on a 40° C hot plate. Each extract was concentrated to approximately 10 ml, and then 1 ml of hexane was added. The extract was further boiled down to 2 ml. At this point, the extract was transferred to an amber sample vial, to which more boiling stones and 0.5 ml hexane were added, and the vial was placed on a 70° C hot plate. The extract was boiled down to 1 ml and removed from the heat in preparation for instrument analysis. The samples were processed for and analyzed by gas chromatography/mass selective detector (GC/MSD) and gas chromatography/flame ionization detector (GC/FID) using NMFS Auke Bay Lab standard operating procedures (Larsen et al. 2003). All GC/MSD and GC/FID analyses were performed by Marie Larsen at NOAA Auke Bay Laboratories in Juneau, AK.

Statistical analysis of data

Two-way Analysis of Variance (ANOVA) (α=0.05) was used to analyzed variation of mortality, swim performance, condition factor, and heart morphology:

$$Y_{ijk} = \mu + O_i + C_j + I_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the *k*th response to oil dose *i* and clutch *j*, μ is the mean response, O_i is the oil dose (a fixed effect), C_j is the clutch (a fixed effect), I_{ij} is the effect due to interaction between oil dose and clutch, ε_{ijk} is random error. The mortality data was arcsine transformed prior to analysis. A one-sample Student's t-test (α=0.05) comparing a treated

batch to the corresponding control batch was used to analyze the pericardial edema and intracranial hemorrhage data.

Results

Water Chemistry

Total PAH concentration in the oiled water dose dropped from 62.1 µg/L on Day 2 to 20.1 µg/L on Day 53 (Fig. 2). Over that time, the concentration of lower molecular weight PAHs such as naphthalenes and fluorene dropped substantially, with some compounds dropping below detection limits (Fig. 3). Higher molecular weight compounds such as dibenzothiophene and some phenanthrenes remained persistent throughout the experiment (Fig. 3).

Water from the unoiled control column had only two samples with any measureable PAHs as determined by GC/MS analysis. Very low concentrations of PAHs appeared in the control water on Day 2 and Day 12 (Fig. 4). The PAH concentrations in the control water consisted of naphthalene only and were more than two orders of magnitude below the concentrations in the oiled water.

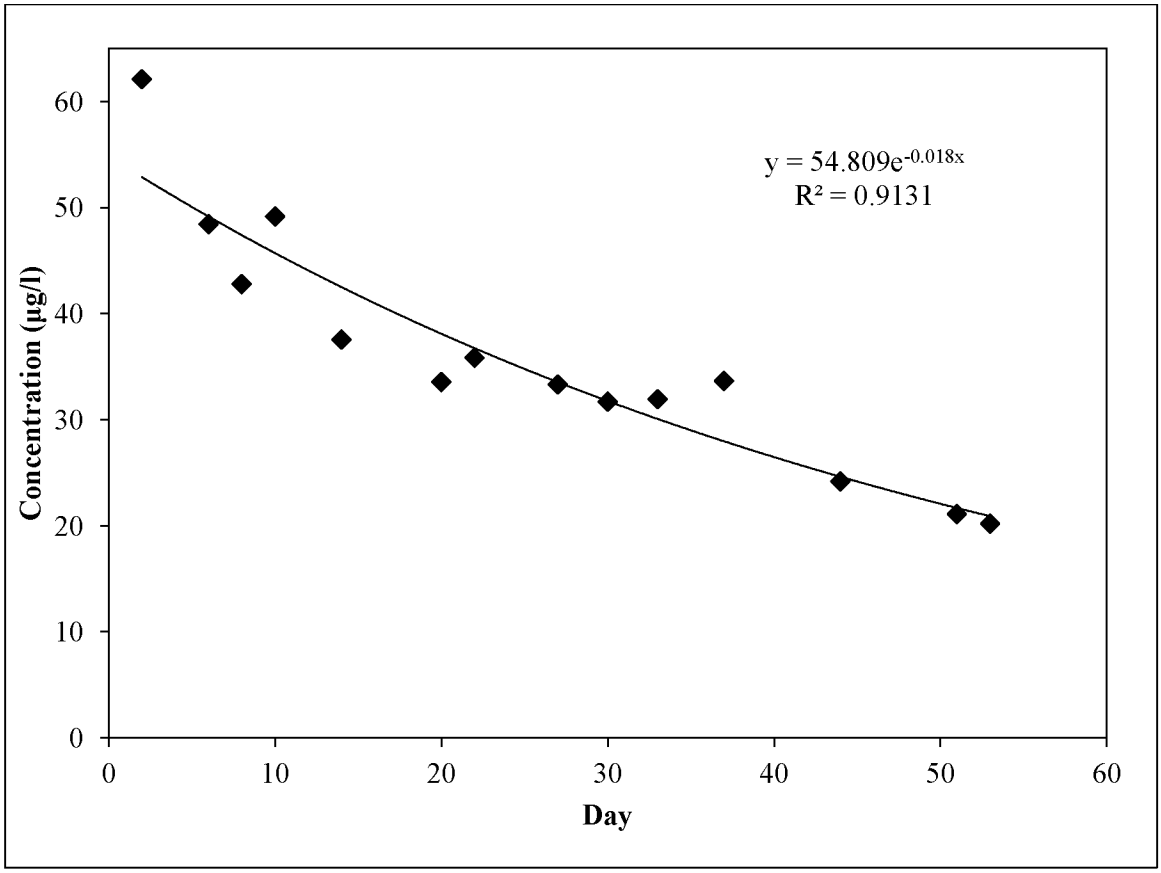

Figure 2. Total PAH concentration of water effluent from the oiled-gravel column over the course of the experiment. The trendline is a best fit exponential regression.

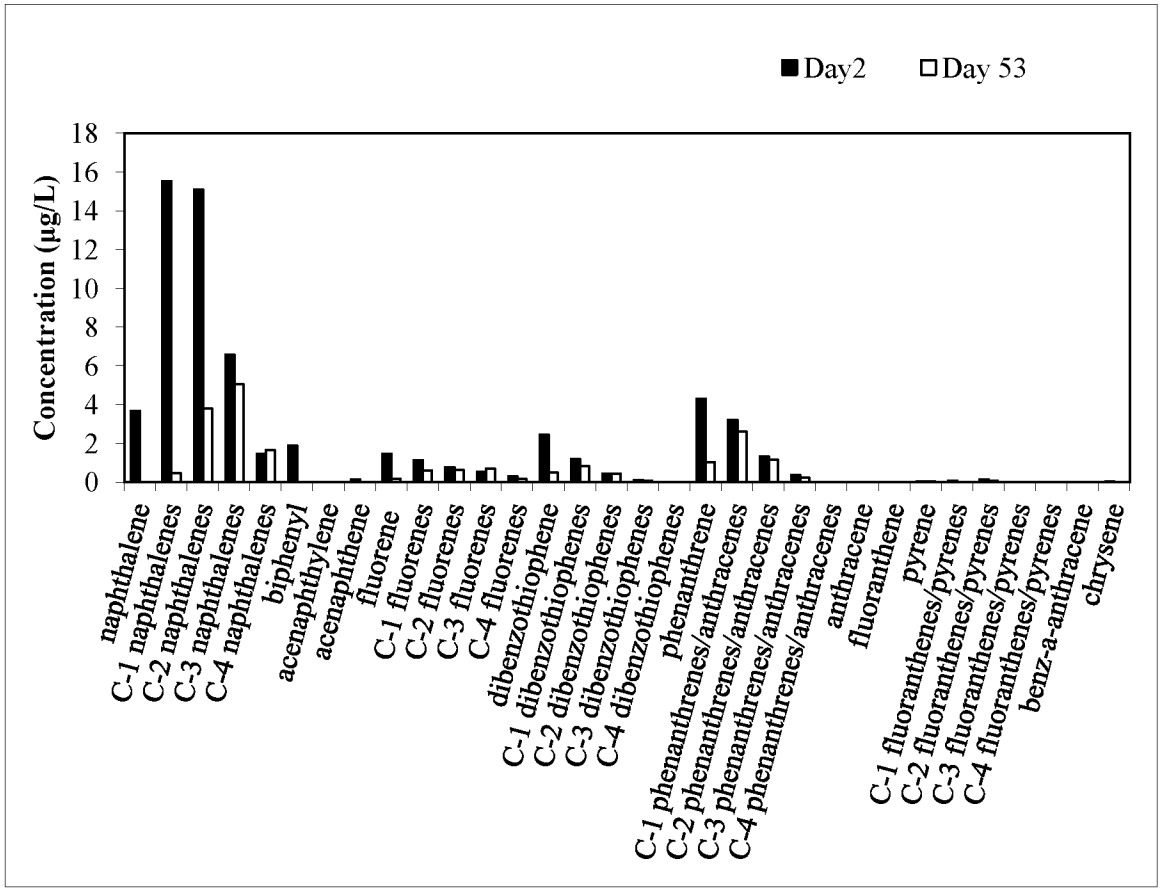

Figure 3. Comparison of individual PAH concentrations in water effluent from the oiled-gravel column between Day 2 and Day 53.

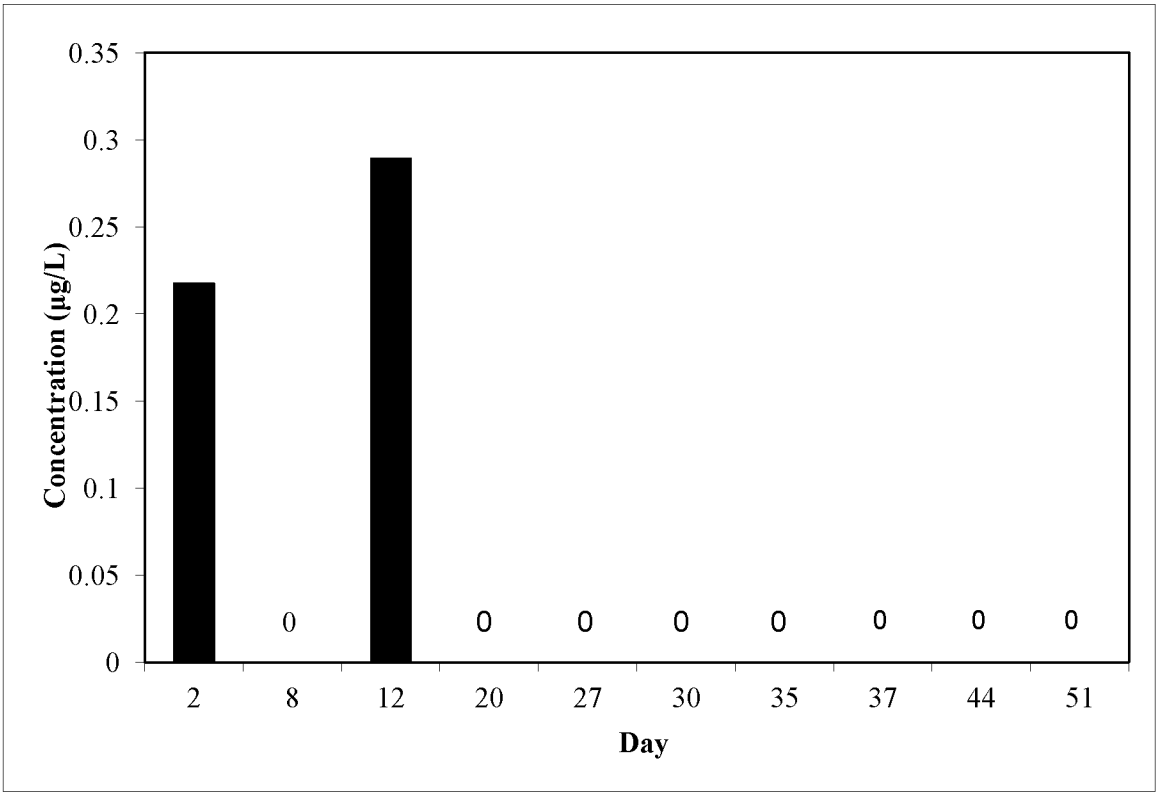

Figure 4. Total concentration of PAHs in water sampled from the control (unoiled) gravel column.

Mortality

Three groups of embryos (Clutch 1, Clutch 2, and Clutch 3 from column days 23, 33 and 44 respectively) were reared to adulthood. The majority of the mortalities occurred after 5 dpf. Mean mortality in these oil exposed embryos varied from 10 to 15% compared to less than 5% for the control fish. Oil exposed embryos had significantly higher mortality compared to the controls (ANOVA, $p \leq 0.003$) (Fig. 5). There was no significant difference in mortality among clutches, and no interaction between clutch and treatment. However, there was a trend of decreasing mortalities as the oil weathered in the column (Fig. 5).

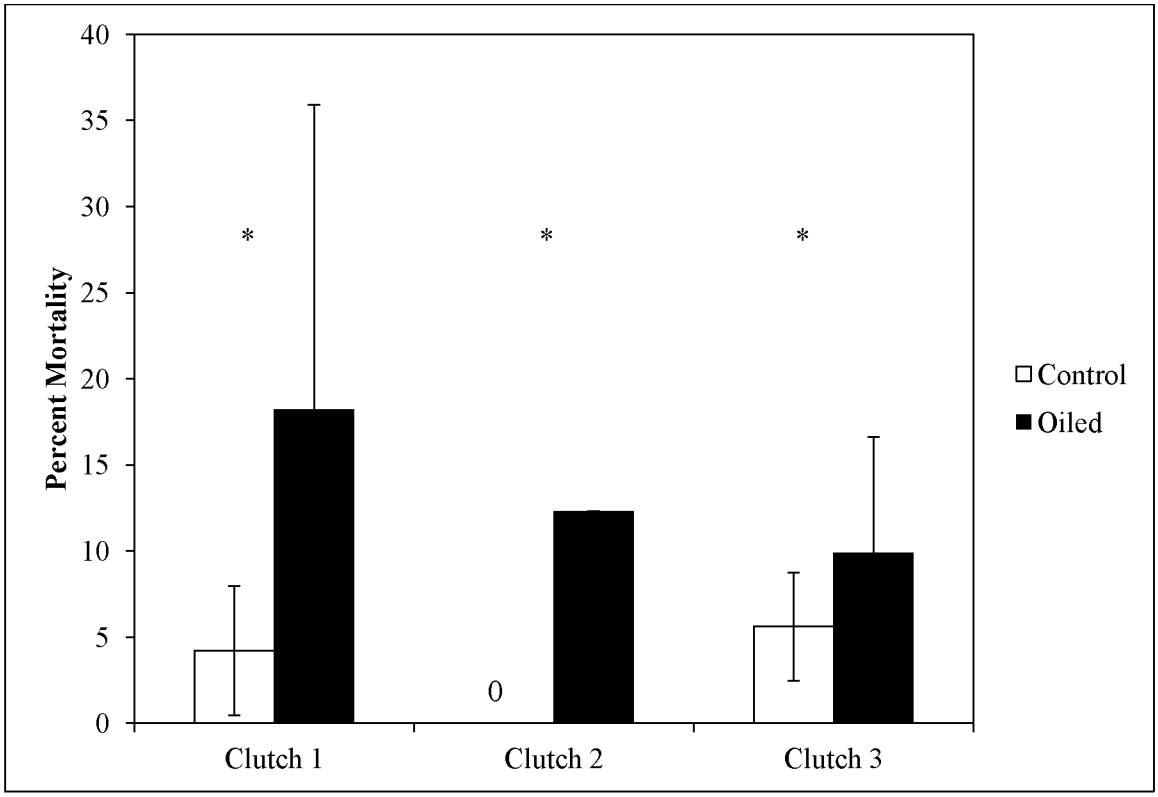

Figure 5. Percent mortality (±95% CI) for three clutches of zebrafish exposed to oiled or control (unoiled) gravel column effluent as embryos and raised to adulthood. Each 48-hour exposure began at a different column age: Clutch 1 on Day 23, Clutch 2 on Day 33, and Clutch 3 on Day 42. Mortality was counted daily for 12 days post fertilization (dpf). The asterisk (*) indicates that for that treatment there is a significant ($P<0.05$) difference between control and previously oiled fish.

Intracranial Hemorrhage and Pericardial Edema

The presence of intracranial hemorrhage and pericardial edema was evaluated 48 hpf for a clutch of embryos exposed to the oiled water column beginning on Day 44 (Fig 6, Fig. 7). The exposed embryos possessed significantly more instances of hemorrhage (Student's t-test, $p\leq0.002$) and edema (Student's t-test, $p\leq0.013$) than their controls (Fig. 8). No control embryo showed evidence of either hemorrhage or edema.

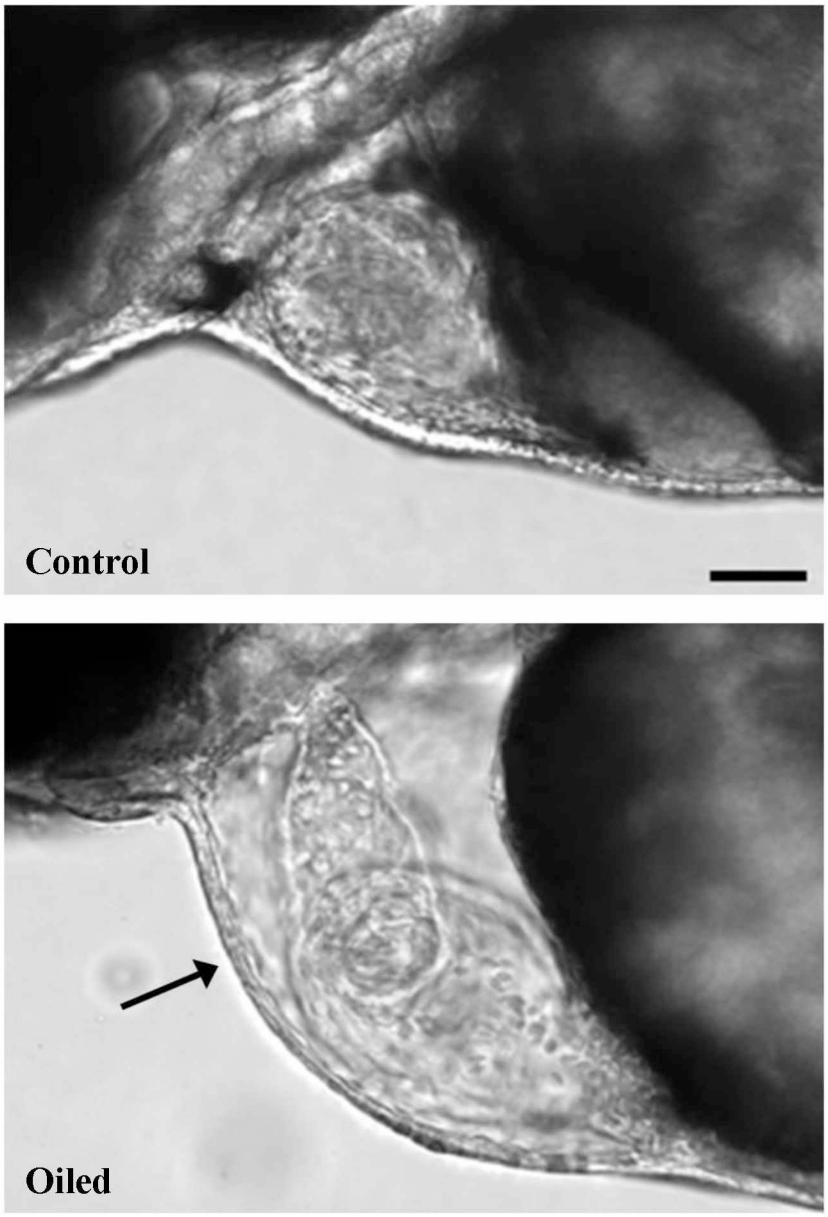

Figure 6. Pericardial edema in an oil-exposed zebrafish embryo as compared to a control (unoiled) embryo. The black arrow indicates the swelling of the pericardium.

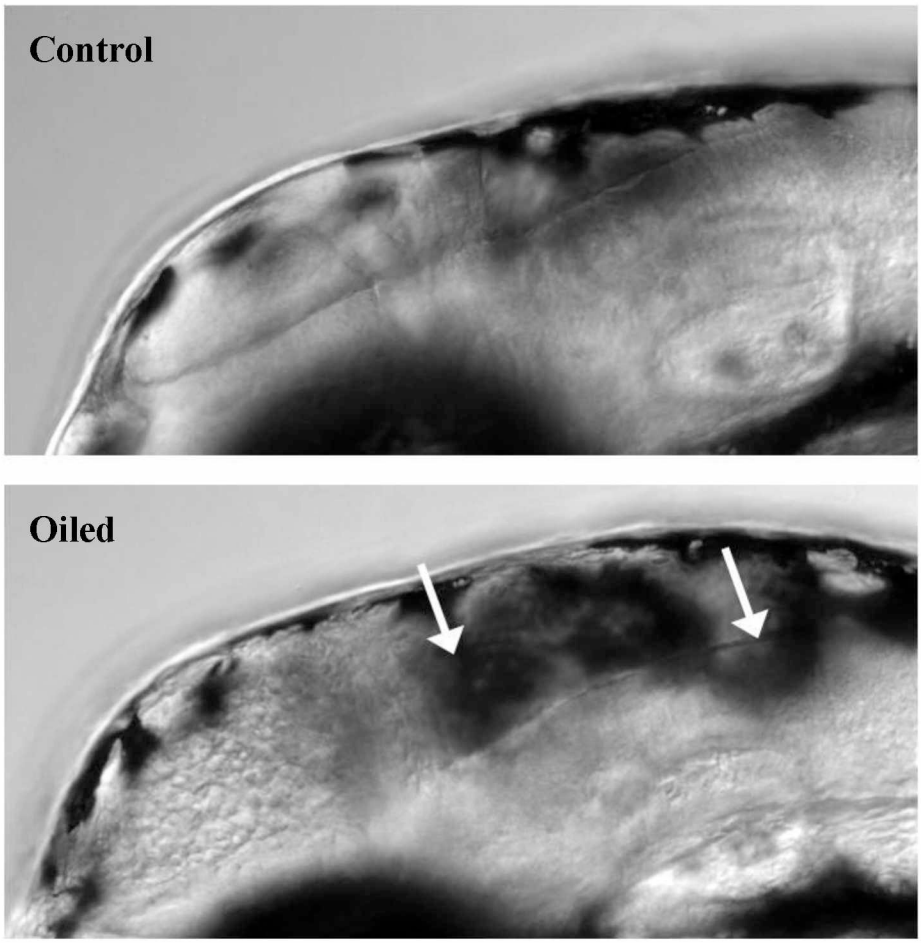

Figure 7. Intracranial hemorrhage in an oil-exposed zebrafish embryo as compared to a control (unoiled) embryo. The white arrows indicate areas of bleeding.

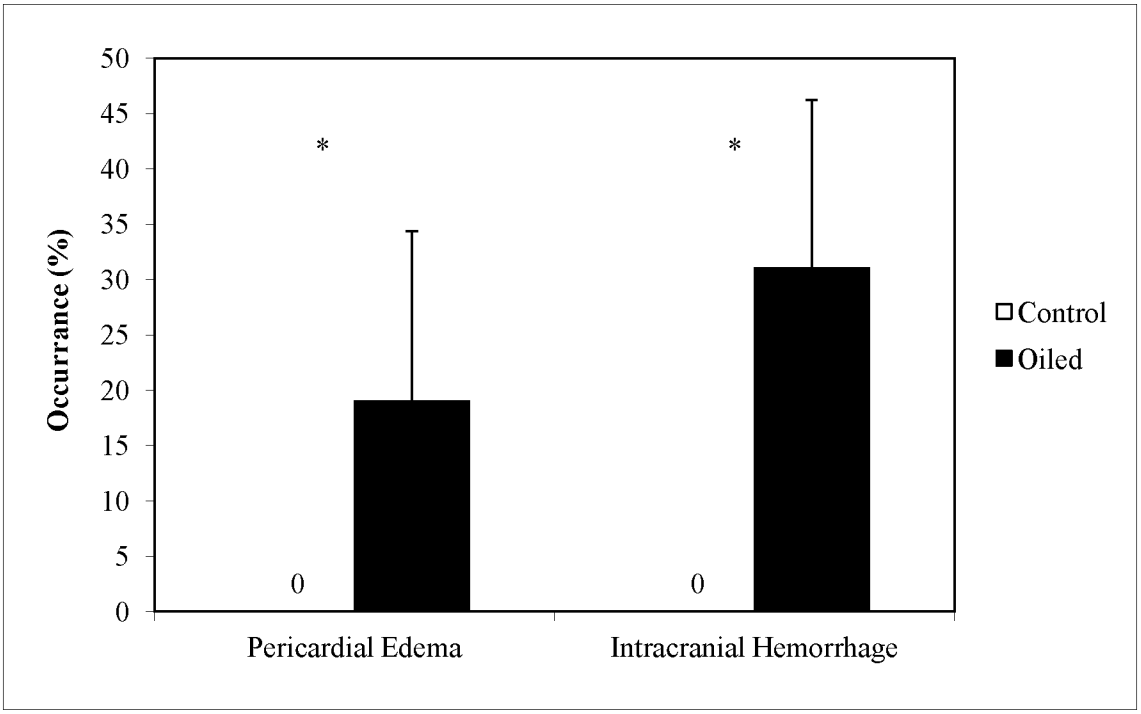

Figure 8. Percent occurrence of pericardial edema and intracranial hemorrhage (± 95% CI) in embryos exposed beginning on Day 44. Each embryo was examined 48 hpf under 20x magnification. The asterisk (*) indicates that for that measurement there is a significant ($P<0.05$) difference between control and previously oiled fish.

CYP1A Immunofluorescence

CYP1A induction indicated that the embryos had been exposed to PAHs, and that the PAHs were taken up by those exposed embryos (Fig. 9). There appears to be more CYP1A expression in exposed embryos than in control embryos (Fig. 9). CYP1A induction was observed in the epithelium, trunk musculature, and heart musculature of the embryos (Fig. 9).

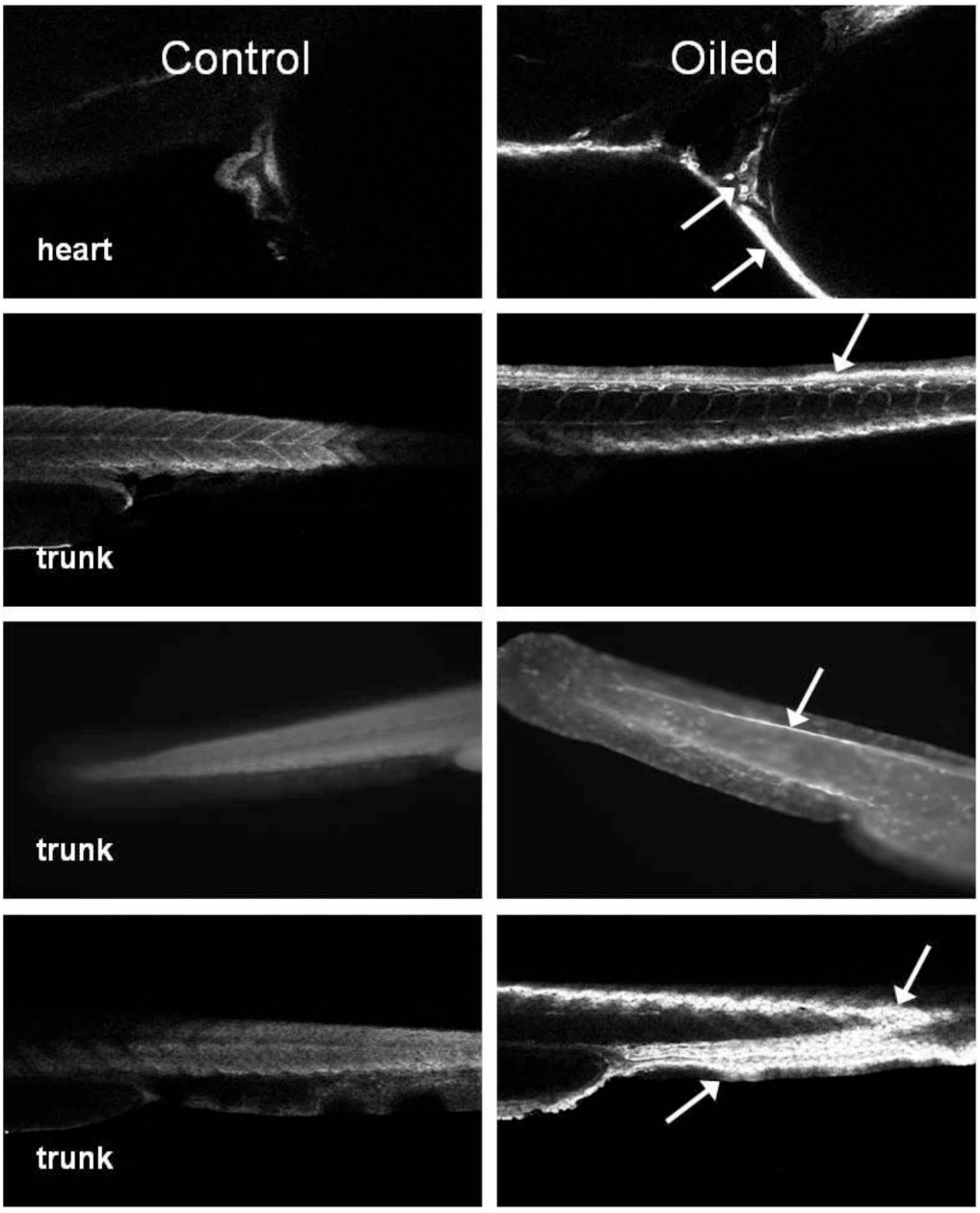

Figure 9. Patterns of CYP1A induction in control and oil-exposed zebrafish. The white areas indicated by the arrows show CYP1A induction as fluorescence in the oil exposed embryos. The presence of CYP1A can be seen in the heart musculature, the epithelium, and the trunk musculature.

Swim Performance

Critical swim speed (body lengths/second) was determined for each of the three clutches raised to adulthood (ages 10-11 months) and compared to their controls. The previously oiled fish (that is, fish that had been exposed to oil as embryos, but subsequently raised in clean water for 10-11 months) had significantly lower critical swim speeds than the controls (ANOVA, $p<0.001$) (Fig. 10). There were no significant differences among the critical swims speed of the clutches, and there was no interaction between clutch and treatment. Critical swim speeds for the previously exposed fish were 11-19% less than that of the controls. Condition factor, which could affect the critical swim speed, was not different between the control and exposed fish (ANOVA, $p\leq0.174$) (Fig. 11). There was no noticeable difference in behavior between control and treated fish in the swim tunnel. In all cases the fish oriented into the current, attempting to maintain their position as the current was increased. When a fish began to tire, it often fell against the screen but recovered when prodded by the electric barrier. Absolute fatigue was determined when a fish could not recover and remained against the screen despite receiving a shock from the electric barrier.

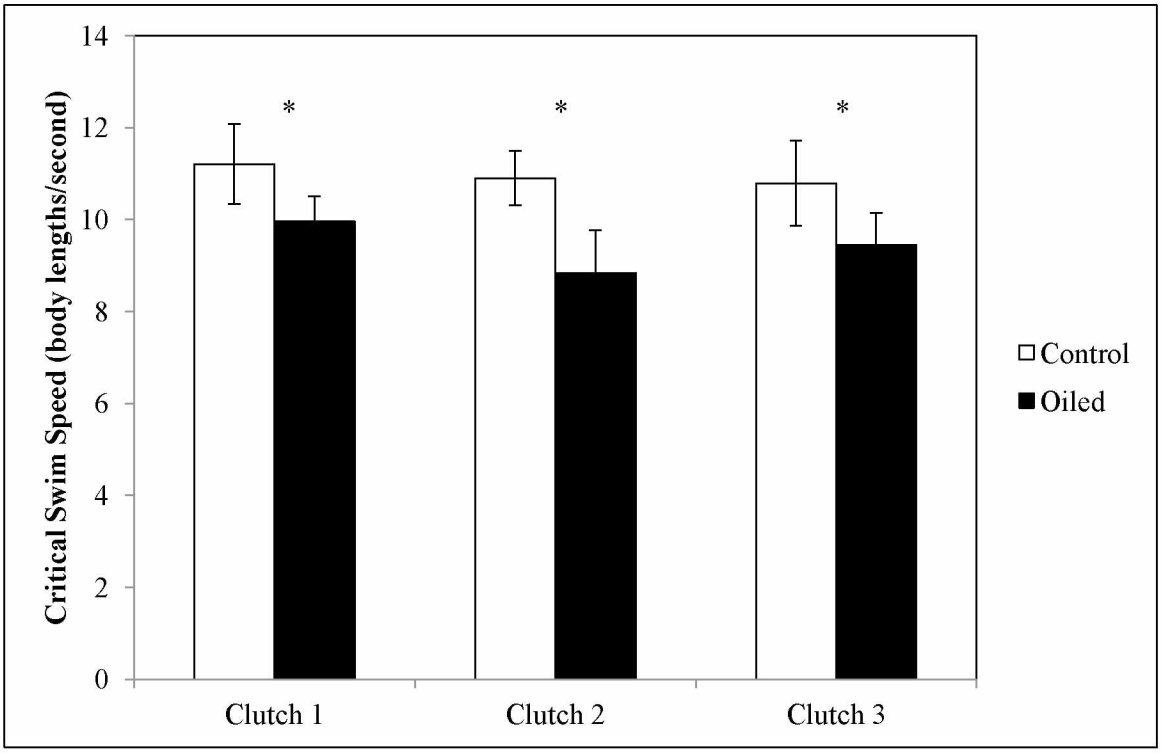

Figure 10. Critical swim speed (±95% CI) of three groups of adult zebrafish exposed as embryos. Each swim trial was run with five fish until the first fish fatigued (n=5 for control, n=9 for oiled). The asterisk (*) indicates that for that treatment there is a significant ($P<0.05$) difference between control and previously oiled fish.

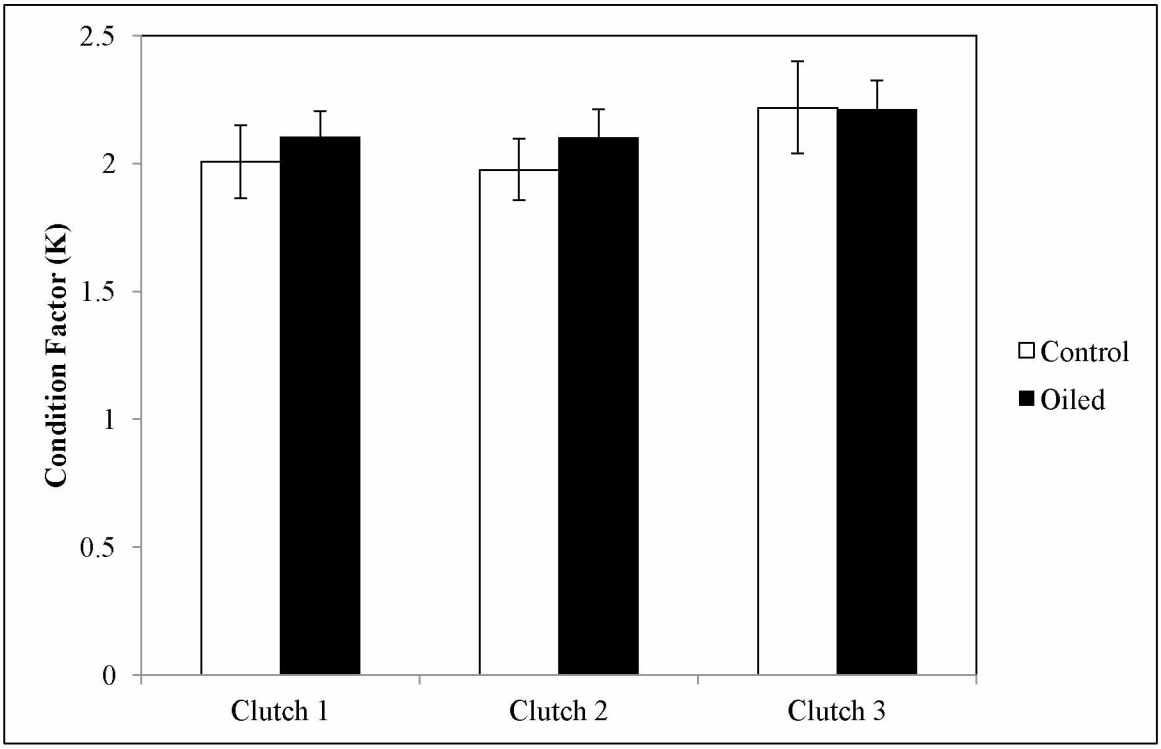

Figure 11. Condition factor (± 95% CI) for control and previously oiled fish. Condition factor was calculated from lengths and weights of fish used in the swim performance trials.

Heart Morphology

The heart ventricle length/width ratios were measured in exposed fish and compared to control fish. The length was measured from the apex of the heart to the ventricular-bulbar valve, and the width was measured at the widest point perpendicular to the length measurement (Fig. 12). The zebrafish exposed to oiled water had significantly rounder hearts than their controls, with an average length/width ratio of 1.42 for exposed fish compared to 1.57 for control fish (ANOVA, $p \leq 0.022$) (Fig. 13).

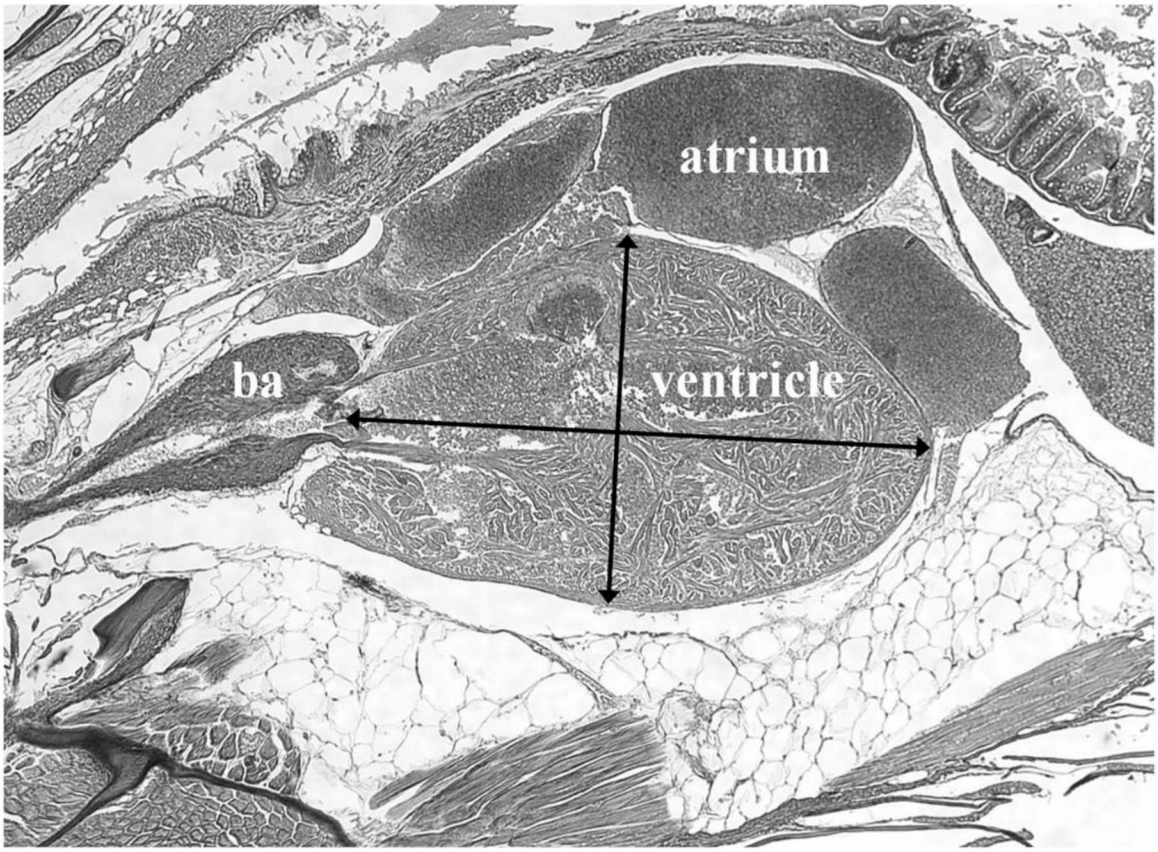

Figure 12. Example of the measurements taken for the heart morphology data (Hicken *et al*. 2011). The length of the ventricle was measured from the ventricular-bulbar valve to the heart apex. The width was measured at the widest point of the ventricle and at 90 degrees to the length measurement.

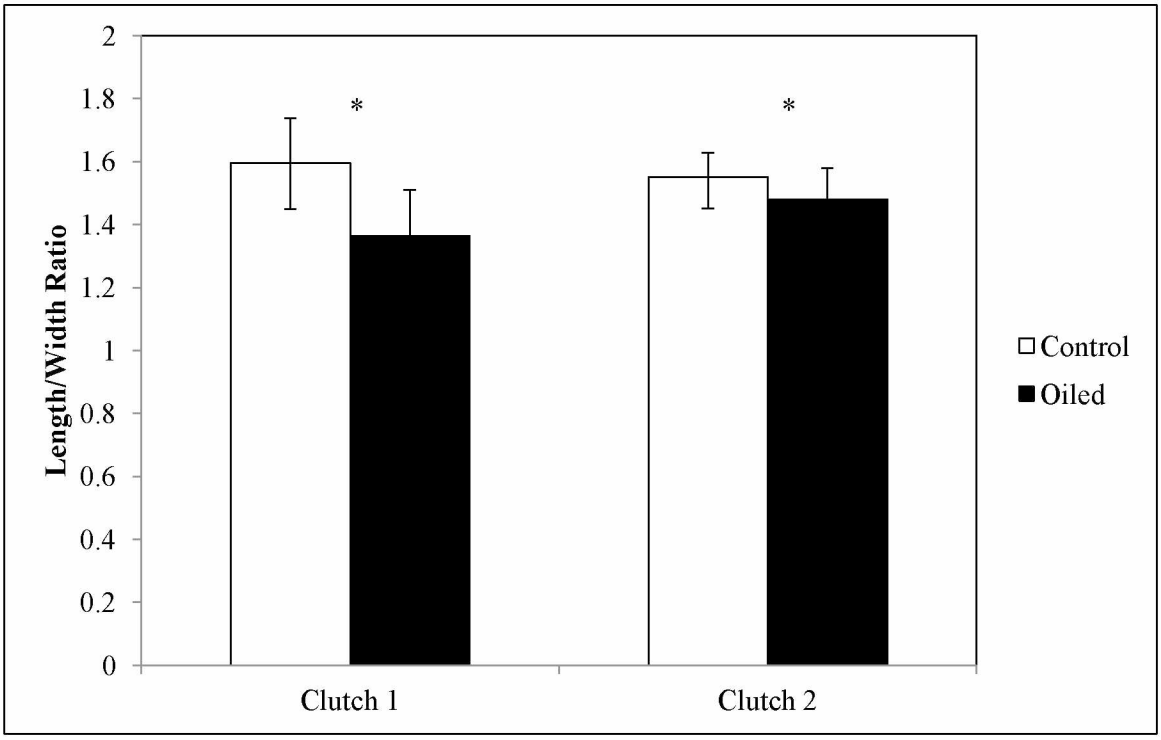

Figure 13. Length/width ratio (± 95% CI) of zebrafish heart ventricles measured from histological sections through the center of the ventricle. The asterisk (*) indicates that for that treatment there is a significant ($P<0.05$) difference between control and previously oiled fish.

Discussion

PAH exposure has long been known to be damaging to the early life stages of fish, causing not only mortalities but also through sub-lethal effects. What is not well understood is how sub-lethal injuries to embryonic and larval fish might cause delayed effects manifested during adulthood, or even deleterious traits in the subsequent generation. These delayed effects could range from minor aberrations to death. Whatever the delayed effects, the relative fitness of the exposed population is likely to be decreased. And decreased fitness will eventually adversely affect the population, especially if the delayed effects are heritable and passed down to subsequent generations never exposed to PAHs.

The objectives of this study were two-fold. We wanted to develop a model system to evaluate the effects of organic pollutants on fish populations, and then use that model to examine the sub-lethal, delayed effects of crude oil on zebrafish adults exposed to PAH-contaminated water during the first 48 hours of embryonic development. Our results show that PAH exposure during vulnerable life stages can cause a suite of lethal and sub-lethal effects with ramifications into adulthood.

Our mortality results were consistent with other studies using zebrafish and other fish species. In this study mortality was examined in three groups of exposed embryos and their controls that were reared to adulthood. Mortality was significantly higher in the exposed embryos compared to the unexposed controls in all three groups. It is likely that mortalities were caused by the presence of PAHs in the exposure groups. Incardona et al. (2004) found that saturating levels of dibenzothiophene, phenanthrene, or pyrene caused 100% mortality in zebrafish embryos. In separate studies both pink salmon and herring embryos had significantly higher mortality when exposed to weathered crude oil (Carls *et al.* 1999; Heintz *et al.* 1999; Carls *et al.* 2005). Other fish species including shortnose sturgeon (*Acipenser brevirostrum*), rainbow trout (*O. mykiss*), and Pacific herring also had increased embryonic or larval mortality when exposed to PAHs or PAH-containing products (Kocan *et al.* 1996; Vines *et al.* 2000; Sundberg *et al.* 2006).

Lethality is a clear injury, unambiguous in its effect. Comparatively, sub-lethal and delayed effects can be subtle but still cause significant damage to fish populations. Embryos in our study exposed to the oiled gravel effluent had higher incidences of pericardial edema and intracranial hemorrhaging compared to their controls. Pericardial edema and intracranial hemorrhaging are both signs of impaired blood flow in a developing embryo. In a previous study herring embryos exposed to weathered crude oil also had significantly higher incidences of pericardial edema and spinal defects (Carls *et al.* 1999). Medaka (*Oryzias latipes*) embryos exposed to PAH metabolites developed circulatory abnormalities including pericardial edema (Carney *et al.* 2008). Pericardial

edema can cause dramatic developmental defects in embryonic zebrafish, including dorsal curvature of the body and reduction of eye and jaw growth (Incardona *et al.* 2004).

For many years CYP1A induction has been known as a bioindicator of PAH exposure (Marty *et al.* 1997; Stagg *et al.* 2000; Incardona *et al.* 2006; Wills *et al.* 2009). PAH exposed zebrafish embryos in this study showed elevated CYP1A expression compared with controls. CPY1A activity was seen in the heart, epidermis, and trunk musculature of exposed embryos. CYP1A is activated through the aryl hydrocarbon receptors 1 and 2 (AHR1 and AHR2) in fish (Schmidt and Bradfield 1996). CYP1A facilitates PAH metabolism by transforming PAHs into a more soluble form which aids in excretion. However, PAH metabolism can generate toxic and mutagenic metabolites (Phillips 1983; Ericson and Balk 2000). For example, pyrene toxicity in zebrafish was found to be partly dependent on metabolism in the liver upon CYP1A induction, likely through a toxic metabolite, though some of its toxicity may act though CYPIA metabolism in the vascular endothelium (Incardona *et al.* 2006). Benz[a]anthracene metabolism mediated by AHR2 caused incomplete cardiac looping and pericardial edema in zebrafish embryos at 48 hpf (Incardona *et al.* 2006). Though they can cause significant damage, the AHR and CYP1A pathways are not the only routes to PAH toxicity in fish embryos. Phenanthrene and dibenzothiophene caused cardiac dysfunction in zebrafish embryos in which CYP1A induction was blocked, suggesting another pathway exists for PAH toxicity (Incardona *et al.* 2005).

Mortality, pericardial edema, intracranial hemorrhage and CYP1A induction were all effects of PAH exposure seen in embryonic and larval zebrafish during the course of this study. These effects appeared during and immediately after exposure. PAH exposure can also cause another suite of effects, ones that are not at once apparent during the exposure, but that can reduce fitness later in life. Pink salmon had reduced growth and lower marine survival when exposed to weathered crude oil as embryos (Heintz *et al.* 2000; Carls *et* al. 2005). Lower growth rates in salmon have been shown to cause size-selective predation (Willette *et al.* 1999). Heritable reduction in reproductive ability was seen in

fathead minnows (*Pimephales promelas*) exposed as embryos to benzo[*a*]pyrene (White *et al.* 1999). These delayed effects would not be apparent in a short-term study, but could have a detrimental effect on the population. Changes in anatomy and physiology can profoundly affect the health and performance of fish, reducing survivability and possibly reproductive success.

Swim performance analyses, including fatigue studies, can be a reliable measure of fish physiological health (Beamish 1978). Juvenile Pacific herring (*C. pallasi*) had reduced critical swim speeds when exposed to a water-soluble fraction of crude oil (Kennedy and Farrell 2006). Swim performance has also been used to examine the effects of other pollutants on fish, including thermochemical newspaper effluent, pulpwood fiber, and pesticides (MacLeod and Smith 1966; Peterson 1974; Linton *et al.* 2005). In this study, fish from three exposure groups were fatigued in a swim chamber. Critical swim speed (U_{crit}) was calculated from the observed time to fatigue, and is a measure of maximum aerobic performance (Brett 1964). U_{crit} was measured for the three groups of PAH exposed zebrafish and their controls that were raised to adulthood. The exposed fish had significantly lower U_{crit} compared to the controls. In the wild adult swim speed can have an important role in survivability and reproduction. Reducing U_{crit} in a migratory fish might compromise its ability to return to spawning grounds (Schreck 1990). A fish with a depressed U_{crit} may be unable to successfully evade predation (Kruzynski and Birtwell 1994). Swimming ability and fitness is critical to the survival and success of most fish populations. Any impairment of swimming ability may cause long-term injury to the health of the population.

We know that PAH exposure can cause a suite of physiological effects in fish, including reduced growth and lower critical swim speeds (Heintz *et al.* 2000; Kennedy and Farrell 2006; Meador *et al.* 2006). PAHs have also been shown to cause many cardiovascular dysfunctions in fish, including heart malformations, irregular heartbeats, and pericardial edema (Incardona *et al.* 2004; Incardona *et al.* 2006). Zebrafish have been used as a model organism for many genetic and development studies, including cardiac

development (Stainer and Fishman 1994; Yelon 2001). Zebrafish embryos are transparent, facilitating observation of the developing heart (Stainier and Fishman 1994). Heart development in zebrafish is also relatively rapid, completed by 48 hours post fertilization. Both of these aspects of zebrafish cardiac development help make it an ideal model for study. In this study, we have identified a possible link between cardiovascular dysfunction and a subsequent reduction in physiological fitness in zebrafish.

Measurements of heart ventricle length and width were made on both control and PAH exposed zebrafish. The ventricles of the exposed zebrafish in this study had significantly smaller length/width ratios compared to control fish. These results suggest that crude oil exposure causes significant effects on cardiac development and morphology in fish. These effects may not be visibly obvious, but they can affect the physiological well-being of the fish. Fish heart development is a closely-matched association of form and function. Any changes wrought in heart form could cause considerable impact on the function of the heart later in life. Studies have demonstrated a connection linking ventricle shape, cardiovascular function, and swim performance (Graham and Farrell 1992; Claireaux *et al.* 2005). Fast swimmers like clupeids and salmonids normally have pyramid-shaped ventricles (Santer *et al.* 1983; Graham and Farrell 1992). Fish within those populations, though, that have more circular hearts, have been shown to have poorer swim performance. Rainbow trout (*O. mykiss*) identified as "poor" swimmers had significantly smaller heart ventricle length/width ratios than trout identified as "good" swimmers (Claireaux *et al.* 2005).

Our results demonstrated that subtle changes in cardiac morphology are associated with significant effects on cardiac performance later in life. Zebrafish exposed to crude oil as developing embryos but raised to adulthood in clean water still showed damage from the initial exposure. This damage was not apparent to the naked eye, but was evident once the fish were put under physiological stress in the swim performance trials. Delayed, sub-lethal effects of PAH exposure to fish could cause lasting damage to the population.

PAHs are introduced to marine and freshwater environments via many different routes. While dramatic events like the *Exxon Valdez* oil spill generate headlines, non point-source pollution such as urban runoff can be damaging as well. While much of the world's coastlines are unlikely to suffer through a major oil spill, many of them are vulnerable to PAH input from non point sources such as automobile exhaust and urban runoff. This input is only increasing as more development occurs. Perch (*Perca fluviatilis*) located on the Swedish Baltic coast showed an increase in PAH metabolites from 2003-2006, indicating an increased presence of those compounds (Hanson *et al.* 2009). An urbanized estuary in the southeastern United States had higher concentrations of PAHs in the seawater and in oyster tissue, as well as lower fish biomass, when compared to a relatively pristine estuary (Vernberg *et al.* 1992). Fish early life stages are susceptible to these chemicals and are often found in lakes, streams and marine intertidal areas, the places where non point-source pollution is worst.

This study shows that a brief PAH exposure to fish at the earliest life stages causes a wide array of effects. Some effects are immediate. Mortality, pericardial edema, and intracranial hemorrhage were all significantly higher in exposed zebrafish embryos compared to controls. CYP1A induction was evident in the heart, epidermis, and trunk musculature of embryos exposed to PAHs, indicating that PAH uptake was occurring during exposure. Some effects were delayed. The zebrafish exposed to PAHs as embryos in this study also showed impairment as adults, long after the exposure had ended. The critical swim speeds of exposed fish were significantly lower than their controls. The exposed fish had significantly smaller heart ventricle length/width ratios. Changes in heart morphology may have contributed to the reduced swim performance shown in the exposed fish.

All of these effects could have a significant impact on the health of fish populations and their ecosystems. Increased mortality through exposure to contaminants can be incredibly harmful to a fish population. For instance, whole brood years could be affected in an area polluted by an oil spill. The subsequent collapse of fish stocks affects

both marine animals dependent on that population as a food source and commercial fishermen dependent on it for their livelihood. The sub-lethal and delayed effects of PAH exposure could be equally damaging to a fish population. Pericardial edema is often seen in concert with jaw malformations and spine curvature in fish (Incardona *et al.* 2004). These defects could reduce growth by impairing feeding abilities, leading to smaller, less healthy fish. These fish would be less likely to reproduce, which could affect the long-term health of the population. Reduced swim performance could cause the fish to be more susceptible to predation, be poorer predators themselves, and less likely to reproduce. These cumulative effects could have far-reaching consequences, both for the fish population and the ecosystem that depends on it.

It is important to understand all we can about the consequences of PAH exposure on fish. There are many questions remaining that could be addressed. What are other effects of PAH exposure in zebrafish? Are growth rates, larval swim speeds, or respiration affected? Would the effects seen in this study be seen in other fish species as well? Are the changes that were seen in zebrafish in this study heritable? White *et al.* 1999 found a heritable reduction in reproductive output and survival in fathead minnows exposed to benzo[*a*]pyrene, though they were not able to control for inbreeding effects. Fish with previous pollutant exposure histories have been shown to develop heritable resistance to the toxicity of those pollutants (Wirgin and Waldman 1998; Meyer *et al.* 2002; Meyer and Di Giulio 2003; Wirgin and Waldman 2004; Wills *et al.* 2009). The heritable resistance in some studies was seen in unexposed F2 generations, suggesting that pollutants like PAHs can cause genetic changes in fish (Meyer and Di Giulio 2003; Wirgin and Waldman 2004). A multi-generation study in which the unexposed offspring of exposed parents are tested for mortality, growth rate, critical swim speed, and examined for heart morphology would determine if the effects of PAH exposure can be passed on to the next and subsequent generations.

Literature Cited

Agnisola, C. and B. Tota. 1994. Structure and function of the fish cardiac ventricle: flexibility and limitations. Cardioscience. 5(3): 145-153.

Bader D., T. Masaki, and D.A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J. Cell Biol. 95(3): 763-770.

Barron, M.G., M.G. Carls, J.W. Short, and S.D. Rice. 2003. Photoenhanced toxicity of aqueous phase and chemically dispersed weathered Alaska North Slope crude oil to Pacific herring eggs and larvae. Environ. Toxicol. Chem. 22(3): 650-660.

Baumann, P.C. and J.C. Harshbarger. 1995. Decline in liver neoplasms in wild brown bullhead catfish after coking plant closes and environmental PAHs plummet. Environ. Health. Per. 103(2): 168-170.

Beamish, F.W.H. 1978. Swimming Capacity. In *Fish Physiology Vol. VIII* (pp. 101-187). Academic Press, Inc.

Berdougo, E., H. Coleman, D.H. Lee, D.Y. Stainier, and D. Yelon. 2003. Mutation of *weak atrium/atrial myosin heavy chain* disrupts atrial function and influences ventricular morphogenesis in zebrafish. Development. 130: 6121-6129.

Brand, D.G., R. Fink, W. Bengeyfield, I.K. Birtwell, and C.D. McAllister. 2001. Salt water-acclimated pink salmon fry (*Oncorhynchus gorbuscha*) develop stress-related visceral lesions after 10-day exposure to sublethal concentrations of the water-soluble fraction of North Slope crude oil. Tox. Path. 29(5): 574-584.

Brett, J.R. 1964. The respiratory metabolism and swimming performance of young sockeye salmon. J. Fish. Res. Board Can. 21: 1183-1226.

Bue, B.G., S. Sharr, and J.E. Seeb. 1998. Evidence of damage to pink salmon populations inhabiting Prince William Sound, Alaska, two generations after the *Exxon Valdez* oil spill. Trans. Amer. Fish. Soc. 127: 35-43.

Carls, M.G., R.A. Heintz, G.D. Marty, and S.D. Rice. 2005. Cytochrome P4591A induction in oil-exposed pink salmon *Oncorhynchus gorbuscha* embryos predicts reduced survival potential. Mar. Ecol. Prog. Ser. 301: 253-265.

Carls, M.G., S.D. Rice, and J.E. Hose. 1999. Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). Environ. Toxicol. Chem. 18(3): 481-493.

Carney, M.W., K. Erwin, R. Hardman, B. Yuen, D.C. Volz, D.E. Hinton, and S.W. Kullman. 2008. Differential developmental toxicity of napthoic acid isomers in medaka (*Oryzias latipes*) embryos. Mar. Poll. Bull. 57(6-12): 255-266.

Cederholm, C.J., M.D. Kunze, T. Murota, and A. Sibantani. 1999. Pacific salmon carcasses: essential contributions of nutrients and energy for aquatic terrestrial ecosystems. Fisheries. 24(10): 6-15.

Claireaux, G., D.J. McKenzie, A. Gaylene Genge, A. Chatelier, J. Aubin, and A.P. Farrell. 2005. Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. J. Exp. Biol. 208: 1775-1784.

Ericson, G. and L. Balk. 2000. DNA adduct formation in northern pike (*Esox lucius*) exposed to a mixture of benzo[a]pyrene, benzo[k]fluoranthene and 7H-dibenzo[c,g]carbazole: time course and dose response studies. Mutat. Res.-Fundam. Mol. Mech. Mutag. 454(1-2): 11-20.

Glickman, N.S. and D. Yelon. 2002. Cardiac development in zebrafish: coordination of form and function. Cell Dev. Biol. 13: 507-513.

Graham, M.S., and A.P. Farrell. 1992. Environmental influences on cardiovascular variables in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish. Biol. 41: 851-858.

Gresh, T., J. Lichatowich, and P. Schoonmaker. 2000. An estimation of historic and current levels of salmon production in the northeast Pacific ecosystem: Evidence of a nutrient deficit in the freshwater systems of the Pacific Northwest. Fisheries. 25(1): 15-21.

Hanson, N., S. Persson, and A. Larsson. 2009. Analyses of perch (*Perca fluviatilis*) bile suggest increasing exposure to PAHs and other pollutants in a reference area on the Swedish Baltic coast. J. Environ. Monit. 11: 389-393.

Heintz, R.A. 2007. Chronic exposure to polynuclear aromatic hydrocarbons in natal habitats leads to decreased equilibrium size, growth, and stability of pink salmon populations. Inter. Environ. Assess. Manag. 3(3): 351-363.

Heintz, R.A., J.W. Short, and S.D. Rice. 1999. Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered *Exxon Valdez* crude oil. Environ. Toxicol. Chem. 18(3): 494-503.

Heintz, R.A., S.D. Rice, A.C. Wertheimer, R.F. Bradshaw, F.P. Thrower, J.E. Joyce, and J.W. Short. 2000. Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. Mar. Ecol. Prog. Ser. 208: 205-216.

Hicken, C.E. T.L. Linbo, D.H. Baldwin, M.L. Willis, M.S. Meyers, L. Holland, M. Larsen, M.S. Stekoll, S.D. Rice, T.K. Collier, N.L. Scholz, and J.P. Incardona. 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc. Nat. Acad. Sci. 108(17): 7086-7090.

Incardona, J.P., H.L. Day, T.K. Collier, and N.L. Scholz. 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol. Appl. Pharm. 217: 308-321.

Incardona, J.P., M.G. Carls, H. Teraoka, C.A. Sloan, T.K. Collier, and N.L. Scholz. 2005. Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. Environ. Health. Per. 113(12): 1775-1762.

Incardona, J.P., T.K. Collier, and N.L. Scholz. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol. Appl. Pharm. 196: 191-205.

Kennedy, C.J. and A.P. Farrell. 2006. Effects of exposure to the water-soluble fraction of crude oil on the swimming performance and the metabolic and ionic recovery post-exercise in Pacific herring (*Clupea pallasi*). Environ. Toxicol. Chem. 25(10): 2715-2724.

Kocan, R.M., MA. Batta, and S.M. Salazar. 1996. Toxicity of weathered coal tar for shortnose sturgeon (*Acipenser brevirostrum*) embryos and larvae. Environ. Contam. Toxicol. 31: 161-165.

Kruzynski, G.M. and I.K. Birtwell. 1994. A predation bioassay to quantify the ecological significance of sublethal responses of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) to the antisapstain fungicide TCMTB. Can. J. Fish. Aquat. Sci. 51(8): 1780-1790.

Larson, M., L. Holland, D. Fremgen, J. Lunasin, M. Wells, and J. Short. 2003. Standard operating procedures for the analysis of petroleum hydrocarbons in seawater, marine sediments, and marine faunal tissue at the Auke Bay Laboratory. Alaska Science Center, Juneau AK.

Linton, E.D., D.A. Scuton, and R.S. McKinley. 2005. Physiological effects of thermomechanical newsprint mill effluent on Atlantic salmon (*Salmo salar* L.). Ecotoxicol. Environ. Safety. 62: 317-330.

MacLeod, J.C. and L.L. Smith, Jr. 1966. Effect of pulpwood fiber on oxygen consumption and swimming endurance of the fathead minnow, *Pimephales promelas*. Trans. Amer. Fish. Soc. 95: 71-84.

Marty, G.D., J.W. Short, D.M Dambach, N.H. Willits, R.A. Heintz, S.D. Rice, J.J. Stegeman, and D.E. Hinton. 1997. Acites, premature emergence, increased gonadal cell apoptosis, and cytochrome P4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. Can. J. Zool. 75: 989-1007.

McKeown, B.A. 1981. Long-term sublethal and short-term high dose effects of physically and chemically dispersed oil on accumulation and clearance from various tissues of juvenile coho salmon, *Oncorhynchus kisutch*. Mar. Environ. Res. 5: 295-300.

Meador, J.P., F.C. Sommers, G.M Ylitalo, and C.A. Sloan. 2006. Altered growth and related physiological responses in juvenile chinook salmon (*Oncorhynchus tshawytscha*) from dietary exposure to polycyclic aromatic hydrocarbons (PAHs). Fan. J. Fish. Aquat. Sci. 63: 2364-2376.

Meyer, J.N. and R.T. Di Giulio. 2003. Heritable adaptation and fitness costs in killifish (*Fundulus heteroclitus*) inhabiting a polluted estuary. Ecol. Appl. 13(2): 490-503.

Meyer, J.N., D.E. Nacci, and R.T. Di Giulio. 2002. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. Toxicol. Sci. 68: 69-81.

Morrow, J.E. 1973. Oil-induced mortalities in juvenile coho and sockeye salmon. J. Mar. Res. 31(3): 135-143.

Moss, J.H., D.A. Beauchamp, A.D. Cross, K.W. Myers, E.V. Farley Jr., J.M. Murphy, and J.H. Helle. 2005. Evidence for size selective mortality after the first summer of ocean growth by pink salmon. Trans. Amer. Fish. Soc. 134: 1313-1322.

Park, S. S., H. Miller, A.V. Klotz, P.J. Kloepper-Sams, J.J. Stegeman, and H.V. Gelboin. 1986. Monoclonal antibodies to liver microsomal Cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): Cross-reactivity with 3-methylcholanthrene induced rat Cytochrome P-450. Arch. Biochem. Biophys. 249: 339-350.

Petersen, G.I. and P. Kristensen. 1998. Bioaccumulation of lipophilic substances in fish early life stages. Environ. Toxicol. Chem. 17(7): 1385-1395.

Peterson, R.H. 1974. Influence of fenitrothion on swimming velocity of brook trout (*Salvelinus fontinalis*). J. Fish. Res. Board Can. 31: 1757-1762.

Phillips, D.H. 1983. Fifty years of benzo[a]pyrene. Nature. 303: 468-472.

Quality Criteria for Water. Environmental Protection Agency. 1986.

Samson, J.C., R. Goodridge, F. Olobatuyi, and J.S. Weis. 2001. Delayed effects of embryonic exposure of zebrafish (*Danio rerio*) to methylmercury (MeHg). Aquat. Toxicol. 51(4): 369-376.

Sanchez-Quintana, D., V. Garcia-Martinez, V. Climent, and J.M. Hurle. 1995. Morphological analysis of the fish heart ventricle: myocardial and connective tissue architecture in teleost species. Ann. Anat. 177: 267-274.

Santer, R.M., M. Greer Walker, L. Emerson, and P.R. Witthames. 1983. On the morphology of the heart ventricle in marine teleost fish (Teleostei). Comp. Biochem. Physiol. 76A(3): 453-457.

Schmidt, J.V., and C.A. Bradfield. 1996. AH receptor signaling pathways. Annu. Rev. Cell Dev. Biol. 12: 55-89.

Schreck, C.B. 1990. Physiological, behavioral, and performance indicators of stress. Amer. Fish. Soc. Symp. 8: 29-37.

Sharp, B.E., M. Cody, R. Turner. 1996. Effect of the *Exxon Valdez* oil spill on the black oystercatcher. Amer. Fish. Soc. Symp. 18: 748-758.

Smoker, W.W., P.A. Crandell, M. Malecha, and I. Wang. 2000. Genetic analysis of development mortality in oiled and unoiled lines of pink salmon. Final Report, Project 40HCNF700235. Juneau Center, School of Fisheries and Ocean Sciences.

Smolders, R., L. Bervoets, G. De Boeck, and R. Blust. 2002. Integrated condition indices as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 21: 87-93.

Spies, R.B., J.J. Stegman, D.E. Hinton, B. Woodin, R. Smolowitz, M. Okihiro, and D. Shea. 1996. Biomarkers of hydrocarbon exposure and sublethal effects of embiotocid fishes from a natural petroleum seep in the Santa Barbara Channel. Aquat. Toxicol. 34: 195-219.

Stagg, R.M, J. Rusin, M.E. McPhail, A.D. McIntosh, C.F. Moffat, and J.A. Craft. 2000. Effects of polycyclic aromatic hydrocarbons on expression of CYP1A in salmon (*Salmo salar*) following experimental exposure and after the *Braer* oil spill. Environ. Toxicol. Chem. 19(11): 2797-2805.

Stainier, D.Y.R. and M.C. Fishman. 1994. The zebrafish as a model system to study cardiovascular development. Trends. Cardiovasc. Med. 4: 207-212.

Stasiunaite, P. 2003. Sublethal effects of heavy fuel oil on rainbow trout (*Oncorhynchus mykiss*) alevins. Ekologija. 2: 28-33.

Steadman, B.L. W.A. Stubblefield, T.W. LaPoint, and H.L. Bergman. 1991. Decreased survival of rainbow trout exposed to No. 2 fuel oil caused by sublethal preexposure. Environ. Toxicol. Chem. 10(3): 355-363.

Stehr, C.M., M.S. Meyers, and M.J. Willis. 1993. Collection of fish tissues for the National Benthic Surveillance Project: Necropsy procedures, tissues processing, and diagnostic procedure for Histopathology. Sampling and analytical methods of the National Status and Trends program, National Benthic Surveillance and Mussel Watch Projects, 1984-1992, NOAA Technical Memorandum NOS ORCA 71 (US Dept. of Commerce, Silver Spring, MD).

Stekoll, M.S., L.E. Clement, and D.G. Shaw. 1980. Sublethal effects of chronic oil exposure on the intertidal clam *Macoma balthica*. Mar. Biol. 57(1): 51-60.

Sundberg, H., R. Ishaq, U. Tharnlund, G. Akerman, K. Grunder, C. Bandh, D. Broman, and L. Balk. 2006. Contribution of commonly analyzed polycyclic aromatic hydrocarbons (PAHs) to potential toxicity in early life stages of rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 63: 1320-1333.

Vernberg, F.J., W.B. Vernberg, E. Blood, A. Fortner, M. Fulton, H. McKellar, and W. Michener. 1992. Impact of urbanization on high-salinity estuaries in the southeastern United States. Neth. J. Sea Res. 30: 239-248.

Vines, C.A., T. Robbins, F.J. Griffin, and G.N. Cherr. 2000. The effects of diffusible creosote-derived compounds on the development in Pacific herring (*Clupea pallasi*). Aquat. Toxicol. 51: 225-239.

Ward, D.L., O.E. Maughan, and S.A. Bonar. 2002. A variable-speed swim tunnel for testing the swimming ability of age-0 fish. N. Amer. J. Aquaculture. 64: 228-231.

Weis, J.S., G. Smith, T. Zhou, C. Santiago-Bass, and P. Weis. 2001. Effects of contaminants on behavior: Biochemical mechanisms and ecological consequences. BioScience. 51(3): 209-217.

Wertheimer, A.C., R.A. Heintz, J.F. Thedinga, J.M. Maselko, and S.D. Rice. 2000 Straying of adult pink salmon from their natal stream following embryonic exposure to weathered *Exxon Valdez* crude oil. Trans. Amer. Fish. Soc. 129: 989-1004

Westerfield, M. 1995. The zebrafish book. Guide for the laboratory use of zebrafish (*Danio rerio*). 3rd ed., Univ. of Oregon Press, Eugene.

White, P.A., S. Robitaille, and J.B. Rasmussen. 1999. Heritable reproductive effects of benzo[a]pyrene on the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 18(8): 1843-1847.

Willette, T.M., R.T. Cooney, K. Hyer. 1999. Predator foraging mode shift affecting mortality of juvenile fishes during the subarctic spring bloom. Can. J. Fish. Aquat. Sci. 56(3):364-376.

Williams, J.E. 2000. The Coefficient of Condition of Fish. Chapter 13 *in* Schneider, James C. (ed.) 2000. Manual of fisheries survey methods II: with periodic updates. Michigan Department of Natural Resources, Fisheries Special Report 25, Ann Arbor.

Wills, L.P., S. Zhu, K.W. Willett, and R.T. Di Giulio. 2009. Effects of CYP1A induction on the biotransformation of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories. Aquat. Toxicol. 92: 195-201.

Wipfli, M.S., J.P. Hudson, and J.P. Caouette. 1998. Influence of salmon carcasses on stream productivity: response of biofilm and benthic macroinvertebrates in southeastern Alaska, U.S.A. Can. J. Fish. Aquat. Sci. 55: 1503-1511.

Wipfli, M.S., J.P. Hudson, J.P. Caouette, and D.T. Chaloner. 2003. Marine subsidies in freshwater ecosystems: salmon carcasses increase the growth rates of stream-resident salmonids. Trans. Amer. Fish. Soc. 132: 371-381.

Wipfli, M.S., J.P. Hudson, J.P. Caouette, N.L. Mitchell, J.L. Lessard, R.A. Heintz, and D.T. Chaloner. 2010. Salmon carcasses increase stream productivity more than inorganic fertilizer pellets: a test on multiple trophic levels in streamside experimental channels. Trans. Amer. Fish. Soc. 139: 824-839.

Wirgin, I. and J.R. Waldman. 1998. Altered gene expression and genetic damage in North American fish populations. Mut. Res. 399: 193-219.

Wirgin, I. and J.R. Waldman. 2004. Resistance to contaminants in North American fish populations. Mut. Res. 552: 73-100.

Woodward, D.F., P.M. Mehrle, Jr., and W.L. Mauck. 1981. Accumulation and sublethal effects of a Wyoming crude oil in cutthroat trout. Trans. Amer. Fish. Soc. 110: 437-445.

Yelon, D. 2001. Cardiac patterning and morphogenesis in zebrafish. Dev. Dyn. 222(4): 552-563.

APPENDIX

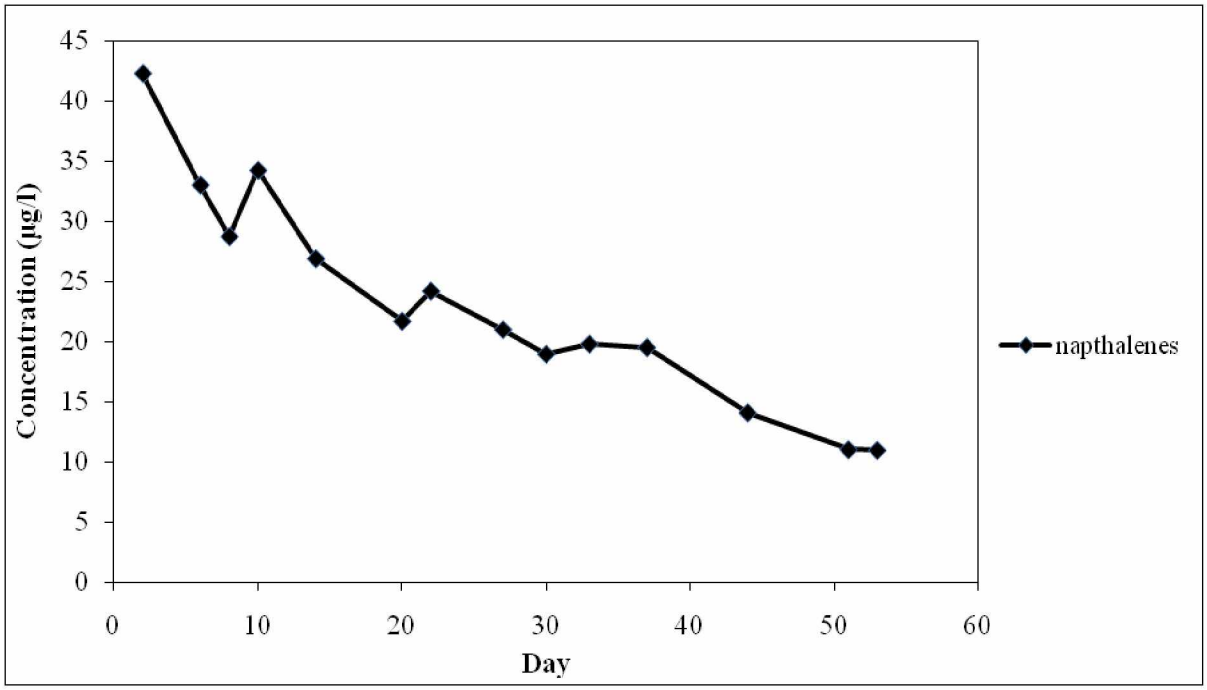

Figure A1. Concentration of all naphthalenes in the oiled gravel effluent over the course of the experiment.

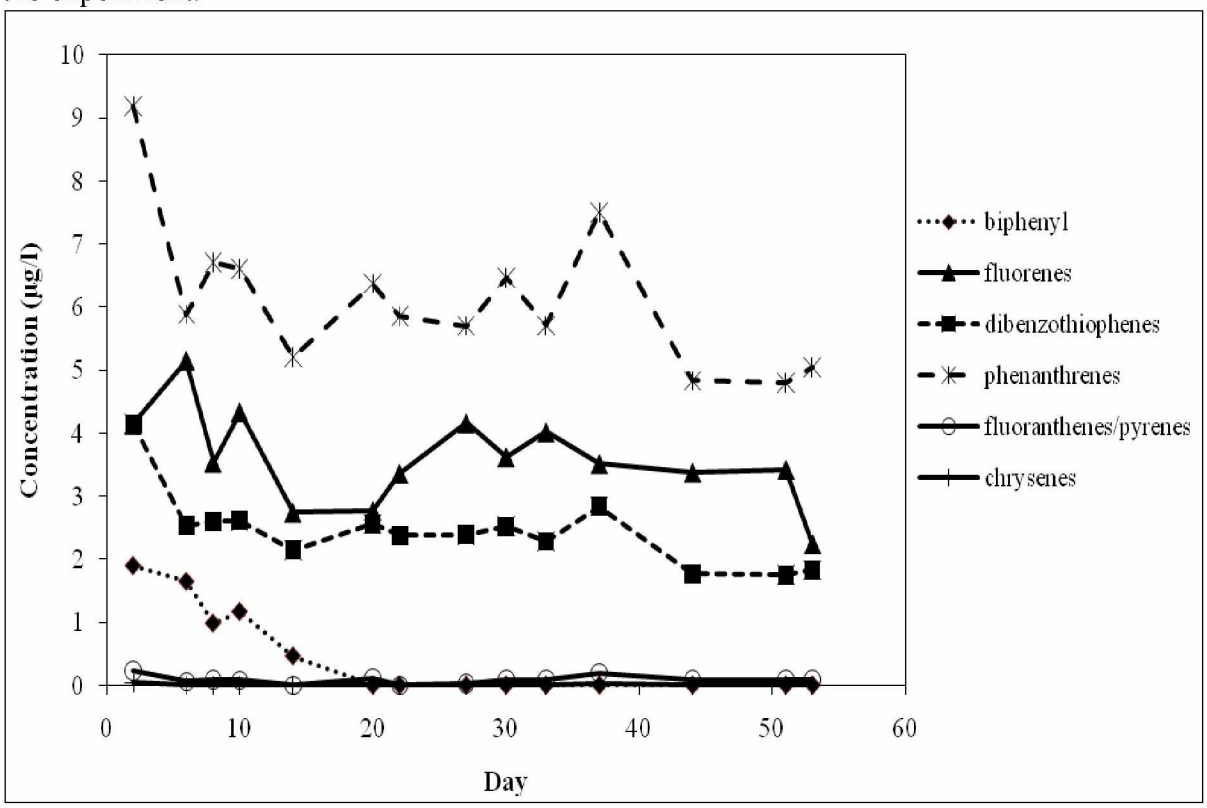

Figure A2. Concentration of all other PAH families in the oiled gravel effluent over the course of the experiment.

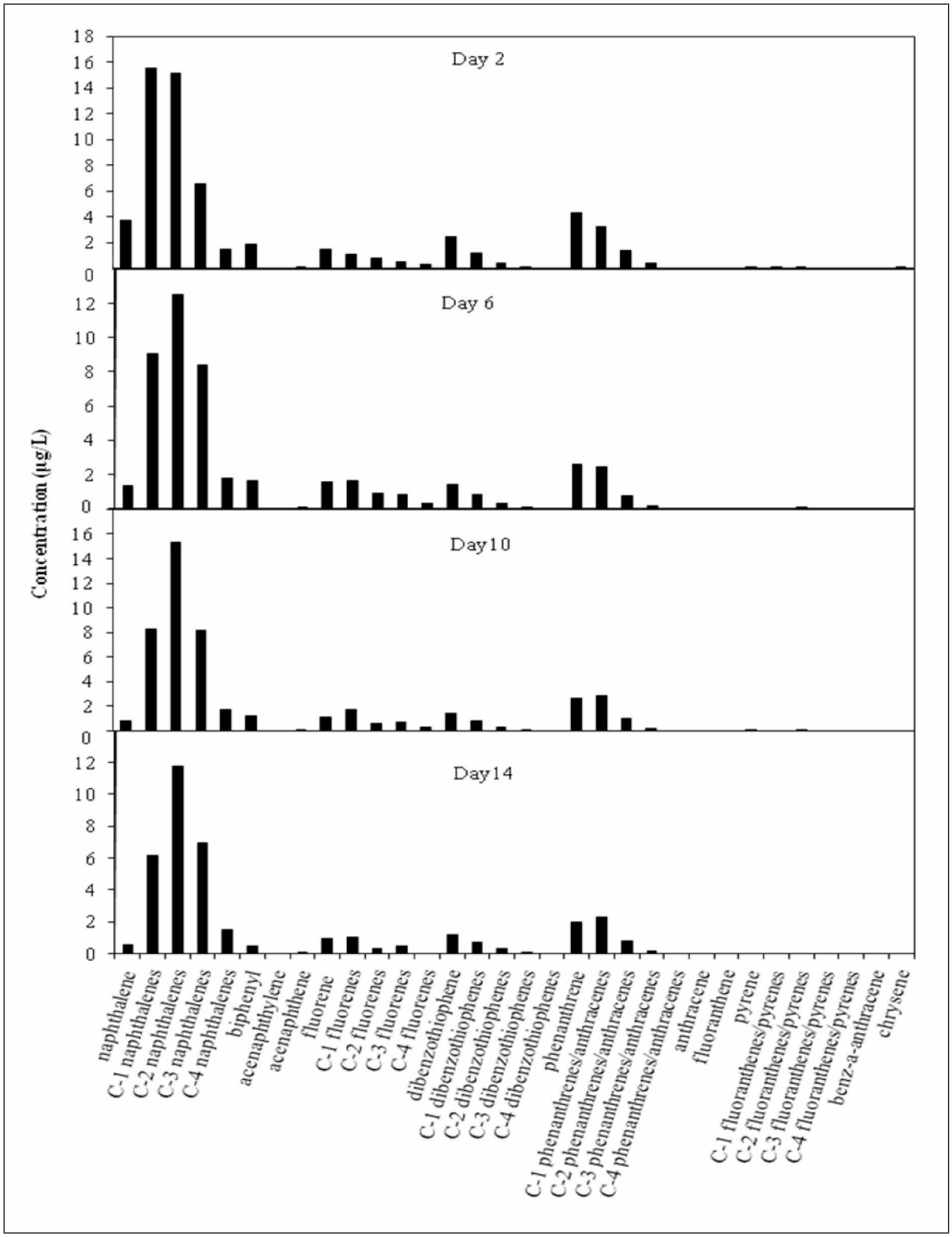

Figure A3. Concentration of PAHs in the oiled gravel effluent on selected days. Note the differing scales of the y-axes.

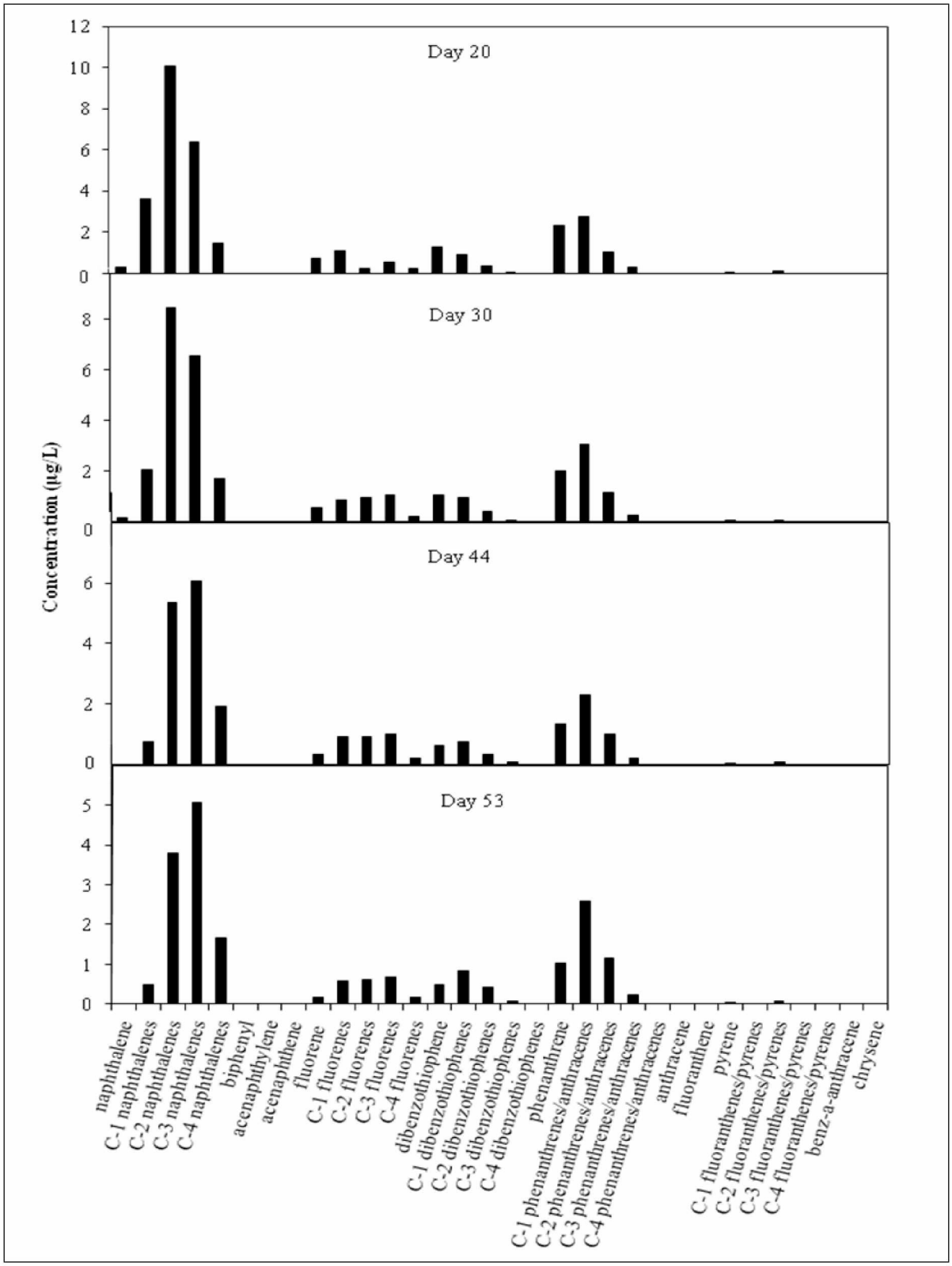

Figure A4. Concentration of PAHs in the oiled gravel effluent on selected days. Note the differing scales of the y-axes.