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Effects of Outboard Motor Emissions on Early Development of the Killifish *Oryzias latipes*

Abstract

Two stroke outboard engines produce large quantities of combustion emissions per liter of fuel consumed. To determine the environmental risk of outboard motor emissions (OME) to the early life stages of fish, we exposed killifish (Medaka), *Oryzias latipes*, embryos to water contaminated with OME. Time for larval development, incidence of larval abnormalities, and larval mortality all increased, and length of hatched larvae decreased, with exposure to increasing concentrations of OME-contaminated water. Chemical analysis using solid phase extraction, followed by gas chromatography, indicated high concentrations of polycyclic aromatic hydrocarbons in the OME water. Application of these results to hypothetical lakes suggests significant risks to fish development in the case of high OME input rates to small lakes.

Introduction

Two-stroke outboard engines are common, yet inefficient, power sources for boats of many kinds. Because these engines combine fuel intake and combustion in the same stroke, some of the incoming fuel-air mixture passes uncombusted through the cylinder. Gases are emitted into the atmosphere and solubilized along with unburned fuel directly into the water (Jackivicz and Kuzminski 1973a). Because of this inefficiency, two-stroke outboard engines are currently being examined as major contributors of hydrocarbons to the environment. Hydrocarbons emitted from outboard engines represent approximately 30% of the total emissions from off-road vehicles (U.S. EPA 1996a). Two-stroke outboard motors produce approximately 200 to 300 g of hydrocarbons per liter of fuel combusted (Tjarnlund *et al.* 1996). Pleasure boats exhaust 1.59×10^9 liters (420 million gallons) of unburned gasoline and other hydrocarbons into U.S. waters each year – a volume equivalent to 40 Exxon Valdez-size oil tanker spills (Mele 1993).

Polycyclic aromatic hydrocarbons (PAHs) are common constituents of combustion emissions and are known carcinogens and mutagens (Neff 1979). They can cause changes in behavioral patterns, growth and reproduction in marine organ-

isms (Connell and Miller 1984). Outboard motor emissions (OME) contaminated water can, in high concentrations, have toxic effects on a variety of adult fish species (Tjarnlund *et al.* 1996). However, little is known about the effects of PAH emissions from outboard engines on embryonic and early life stage development of fish (Jackivicz and Kuzminski 1973b).

Until recently outboard engines were not subject to emission regulations (Mele 1993). However, beginning in December, 1997 the U.S. EPA imposed air pollution standards on recreational watercraft. Tighter standards will be phased in over a period of ten years and will result in more efficient, cleaner-burning, direct fuel injection engines. Nevertheless, with an estimated 120 million two-stroke outboard engines currently in use in the United States (Mele 1993), the PAHs emitted into aquatic environments will likely remain substantial.

Because of the known toxicity of PAHs to aquatic life (Payne *et al.* 1988, Connell and Miller 1984, Neff 1979), we hypothesized that OME would adversely effect the development of fish larvae. We investigated the effects of OME-contaminated water on the development of killifish (Medaka), *Oryzias latipes*. We also measured the PAH content of the OME-contaminated water, and applied our laboratory findings to assess the environmental risk to lakes.

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Methods

Exposure Water

We collected water 1 to 2 days prior to our exposure experiments, from Pass Lake, Fidalgo Island, Washington, which is a relatively pristine lake where outboard engine use is prohibited. A 1968 Evinrude model 3836 three horsepower, two-stroke outboard engine with a fuel mixture of regular unleaded gasoline and two cycle motor oil (50:1) was used for the study. To determine if outboard emissions could cause any toxic effects in the test organism and to define a range of outboard emission concentrations to be used in a definitive experiment, we conducted a pre-test. The outboard engine was operated at medium throttle in a polyethylene tank containing 129 L of lake water for 30 minutes. This volume of water and run time was chosen simply for convenience. Based on toxicity test results from the pre-test (below), we decided to use a 60 minute run time and same volume of water (129 L) in the definitive experiment. To determine fuel burned, the fuel tank was weighed before and after operation. The control and OME contaminated waters were filtered through a 750 μm mesh nitex screen to remove large particles. The control (untainted lake) water was used to dilute the OME water to six water soluble fractions (WSF)-100%, 80%, 60%, 40%, 20%, 0%. To prevent fungal and bacterial growth methylene blue dye was added to each dilution at a concentration of 0.1 ml L⁻¹.

Toxicity Test

We used the killifish *Oryzias latipes* as a test organism because it readily reproduces in the laboratory, has been used in many toxicological studies, and the developmental stages of the transparent embryo have been well documented. Embryos are 1.5 mm in diameter. The larvae are 4 to 5 mm in length at hatching, which occurs in about eleven days at their optimum temperature of 25 °C. They begin feeding about 24 h after hatch (Kirchen and West 1969). We obtained healthy killifish embryos, approximately 29 h post-fertilization, from Carolina Biological Supply Co. and exposed them to 10 ml of each WSF in 15 ml Corning sterile polystyrene cell well plates. The wells were placed in a 25 \pm 1 C incubator with a twelve h light:dark cycle (Kirchen and West 1969).

In the pre-test, to define a range of OME concentrations to use in toxicity tests, we exposed only one embryo per WSF concentration. In the definitive test, to better define the dose response, we used six embryos per WSF concentration. To establish a well-defined baseline 12 embryos were used per control. All results presented are based on the definitive test.

Daily observations of the embryos were made with a dissecting microscope at 16x, 25x, and 40x. We recorded data on heart rate, pigmentation, fin and organ development, and movement. Death was determined by the absence of a heartbeat. Developmental stages (Kirchen and West 1969) were noted until approximately 125 h post-fertilization. Within 8 h post-hatching, the larvae were preserved in 5% formalin and within 7 days larval length was measured using an IBM-PC Optimas program and a Sony VCC Video camera, calibrated to a Wild 16x lens.

Chemistry

Outboard motor emissions water (100% water-soluble fraction, WSF) was analyzed using U.S. EPA Method 525 (Supelco 1995, Eichelberger *et al.* 1988). Solid phase extractions were performed using Supelco Inc. ENVI™-18 cartridges and J.T. Baker® Ultra-grade resi-analyzed toluene and methanol. Each column was conditioned with 2 X 6 ml of toluene:methanol (10:1) followed by 6 ml methanol and 6 ml nanopure H₂O. Ten ml of 100% WSF were extracted at 3 to 4 psi and a flow rate of 5 ml min⁻¹ followed by 15 min of air to dry the sample. Samples were eluted with 2 X 1 ml of toluene:methanol (10:1) and refrigerated at 3 to 4°C for approximately 24 h prior to analysis. Samples were analyzed using a Hewlett-Packard 5890 gas chromatograph. Sample solvent blanks and a standard solution containing 10 ppm of 16 different PAHs were run under the same conditions and used to identify and quantify unknown peaks present in the OME samples.

Results

Toxicity Test

After 211 h of exposure, all control embryos had hatched. Delayed hatching was observed in the higher WSFs (Figure 1). Delays in embryo development were evident as early as 18.5 h after initial exposure to OME water (45.5 h post-fertilization). Final

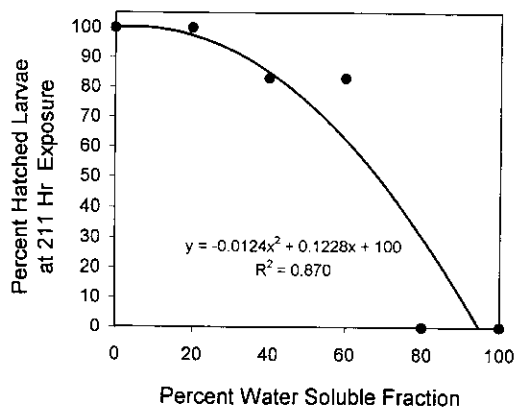


Figure 1. Relationship between % WSF of OME and % larval hatch after 211 hours exposure.

hatch time in 80% WSF was 353.5 hours post-exposure, i.e. 142.5 hours later than complete hatching in the 0% WSF.

Numerous morphological abnormalities occurred in embryos or larvae exposed to OME (Table 1). Effects included underdeveloped or abnormal eyes, absence of spleens or swim bladders, spinal abnormalities, underdeveloped hearts, and mortality. In WSFs of 60% or greater embryonic eyes lacked visible pupils. Spleens, easily visible in the control fish, were frequently absent in larvae exposed to 20% or greater WSF (Table 1). Kyphosis (abnormal spinal curvature) occurred in 50% of embryos exposed to 40% or greater WSFs. In addition, irregular or slowed heart rates were observed in water-soluble fractions (WSFs) of 60% and higher. The length of newly hatched larvae decreased with increasing WSFs of OME (Figure 2). At 172 h, 17% of embryos in 60%

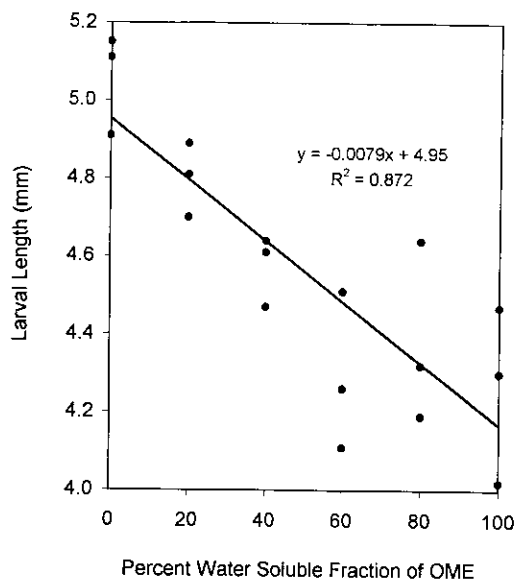


Figure 2. Larval length decreases with increasing WSF.

WSF died (Table 1) and overall mortality increased with an increasing percent WSF (Figure 3).

Chemistry

After operating the engine, the tank water had a strong odor of oil and gasoline, appeared cloudy, and was tan in color. The solvents eluted from the cartridges in two phases and the top organic layer was run through the gas chromatograph. Despite this phase separation, recovery of PAHs from the standard solution was 90% or greater with low background noise in the untainted water sample.

TABLE 1. Percentage embryo/larval abnormalities following exposure to different water soluble fractions (WSF) of outboard motor emissions. For each WSF n=42, ND=no data

Abnormality	Percent Water Soluble Fraction of Outboard Motor Emissions					
	0	20	40	60	80	100
Dead prior to hatching	0	0	0	17	ND	67
Dead following hatching	0	0	0	ND	100	33
Abnormal heart	0	0	17	17	83	100
Loss of blood color	0	0	17	50	100	100
Abnormal swimming	0	17	0	83	ND	ND
Curved spine	0	0	50	50	83	ND
Abnormal eyes	0	17	0	100	ND	67
Absence of swim bladder	0	0	50	67	ND	ND
Absence of spleen	0	33	83	83	ND	ND

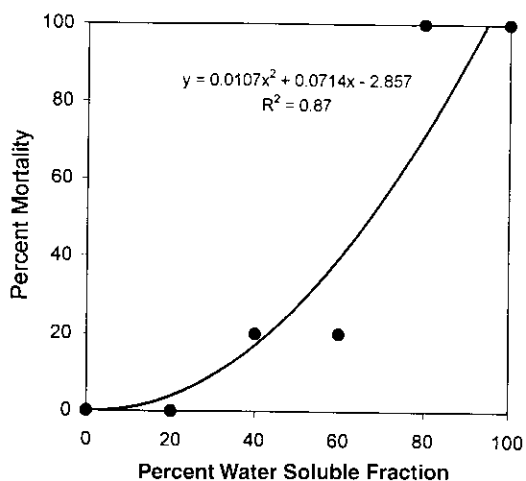


Figure 3. Percent larval mortality increases with increasing WSF of OME.

The OME samples contained numerous highly concentrated PAHs. Because of the complexity of the mixture and overlapping peaks, only four specific PAHs were identified in the OME water: Naphthalene (N), Acenaphthene (A), Benzo[k]fluoranthrene (BkF), and Benz[a]anthracene (BaA). Concentrations of PAHs in the 100% WSF were 180 to 5000 $\mu\text{g L}^{-1}$ (Figure 4).

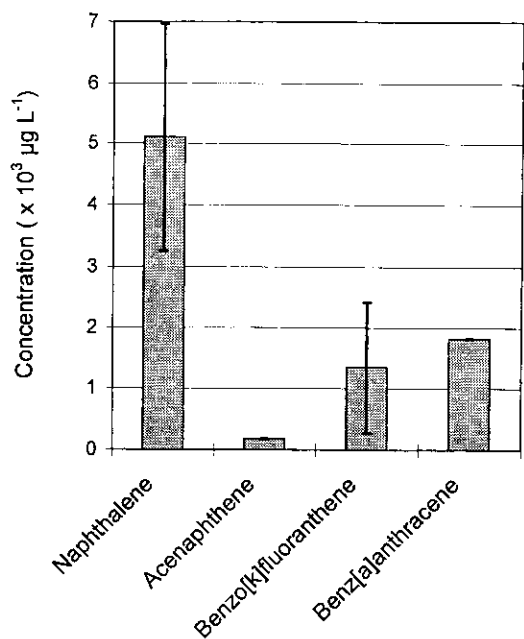


Figure 4. Four PAHs and their concentrations in 100% WSF.

Discussion and Environmental Risk

We found numerous adverse developmental effects in killifish exposed to outboard motor emission (OME) water. Our data clearly indicate a delay in development time of embryos exposed to OME water at all water-soluble fraction concentrations. Levels of PAHs in the 20% or greater WSFs undoubtedly account for the mortality at hatching and other adverse effects observed in our study. Assuming similar compound solubility, observed effects would be at concentrations of about 1000 $\mu\text{g L}^{-1}$ N, 360 $\mu\text{g L}^{-1}$ BaA, 280 $\mu\text{g L}^{-1}$ BkF, and 36 $\mu\text{g L}^{-1}$ A. Other studies report biological effects at similar concentrations. For example, naphthalene is toxic to pink salmon fry at 920 $\mu\text{g L}^{-1}$ (Neff 1979), and soluble aromatic derivatives are lethal to marine larval stages at concentrations of 100 to 1000 $\mu\text{g L}^{-1}$ (Connell and Miller 1984). Acenaphthene at a concentration of 608 $\mu\text{g L}^{-1}$ causes 50% mortality in the early-life-stage of fathead minnows (Cairns and Nebeker 1982).

Chemical products of outboard motor emissions have been found in aquatic environments. Trace constituents of outboard motor oils and exhausts (certain C27 to C35 pentacyclic terpanes) were found in the plankton of Conception Bay, Newfoundland, Canada (Bieger et al. 1996). Little information exists on concentrations of PAHs in lake water. Concentrations of total PAHs were .025 to 0.234 $\mu\text{g L}^{-1}$ in Lake Constance Germany (Borneff and Kunte 1964) and .005 $\mu\text{g L}^{-1}$ in Lake Erie at Buffalo, New York (Basu and Saxena 1978).

Hydrocarbons of low solubility, including PAHs, accumulate in surface films (the aquatic surface microlayer) where they reach high concentrations (Hardy 1987, Hardy and Cleary 1992). These films pose a threat to surface-living and surface-feeding organisms (neuston) (Hardy et al. 1987) and, through wind and current patterns, deposit on beaches (Gardiner 1992).

Furthermore, shoreline residents on some lakes draw their drinking water directly from the lake. The U.S. EPA has established drinking water standards for only a few PAHs (EPA 1996b). For benzo[a]pyrene (BaP) the EPA standard is 0.2 $\mu\text{g L}^{-1}$, and in Washington State the standard for groundwater is 0.008 $\mu\text{g L}^{-1}$ (WAC-173-200-040[2]). For naphthalene, the drinking water equivalent level (DWEL) is 100 $\mu\text{g L}^{-1}$.

The PAH concentrations found in our study resulted from one small (3 horsepower) outboard engine burning 0.5 liters of fuel in a 129 L tank. What might be the effects of multiple engine emissions on fish development in a real lake? The concentration of PAHs in lake water resulting from outboard emissions will be the result of many interacting factors, but can be approximated as follows:

$$\text{PAH } \mu\text{g L}^{-1} \text{ lake water} = \frac{(\text{number engines})(\text{average use h day}^{-1})(\text{L fuel consumed engine}^{-1} \text{ h}^{-1})(\mu\text{g soluble PAH produced L}^{-1} \text{ fuel consumed})(\text{residence time of PAH, days})}{(\text{lake volume L})}$$

Using this model, we predict resultant PAH concentrations and fish larval toxicity based on several scenarios of boat use and lake volume, assuming negligible inflow/outflow dilution (Table 2). Fuel consumption for outboard engines differs greatly, but for a moderate-sized engine we assume 30 liters h⁻¹. In Table 2, quantities of PAH emitted L⁻¹ fuel consumed are based on our chemical analytical results. Lifetimes of PAHs are derived from the literature. For example, PAHs are biodegraded in natural waters with half lives of from 0.5 to 20 days (naphthalene) and 57 to 529 days (benzo[a]pyrene) (Howard et al. 1991). The turnover time of PAHs in a controlled ecosystem is 10 days for naphthalene and 1400 days for benzo[a]pyrene (Neff 1979).

As indicated (Table 2), with 60 outboards, averaging 8 hours per day on a lake of 1 x 10¹⁰ liters volume, no toxic effects on killifish larvae

or on drinking water criteria for naphthalene would be expected. Thirty-five outboards on a lake of 1 x 10⁹ liters, or less would result in naphthalene concentrations exceeding EPA drinking water criteria, but would not likely be toxic to killifish larvae. However, the same 35 outboards on a very small (1 x 10⁸ L) lake would exceed drinking water criteria for naphthalene and would also be toxic to killifish larvae (based either on naphthalene or benzo[k]fluoranthrene concentrations). These estimates do not include the possible additive or synergistic effects of the many other PAHs and chemicals resulting from OME emissions.

In summary, our results suggest that moderate use of two-cycle outboard motors on large lakes poses little or no threat to water quality. However, heavy use of such motors on small lakes with low inflow/outflow dilution could result in PAH concentrations great enough to inhibit early life stage development in some fish. Also, on some lakes PAHs from outboard motors could threaten drinking water quality.

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TABLE 2. Scenarios of lake contamination from outboard motor emissions.

	Contaminant					
	naphthalene			benzo[k]fluoranthrene		
Lake volume (L)	1.00E+10	1.00E+09	1.00E+08	1.00E+10	1.00E+09	1.00E+08
Number engines on lake	60	35	35	60	35	35
Average use (hours day ⁻¹)	8	8	8	8	8	8
Fuel consumption rate (L engine ⁻¹ h ⁻¹)	30	30	30	30	30	30
Soluble PAH (μg L ⁻¹ fuel consumed) ¹	1.29E+06	1.29E+06	1.29E+06	2.80E+03	2.80E+03	2.80E+03
Residence time of PAH (days)	10	10	10	1400	1400	1400
Resulting PAH concentration (μg/L)	19	108	1084	6	33	329
Exceeds toxic level for killifish larvae ²	no	no	yes	no	no	yes
Exceeds EPA Drinking Water Criteria ²	no	yes	yes	NA	NA	NA

¹Our data

²DWEL Drinking Water Equivalent Level. A lifetime exposure concentration protective of adverse, non cancer health effects, that assumes all exposure to a contaminant is from a drinking water source.

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