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A CONTINUOUS FLOW BIOASSAY METHOD TO EVALUATE THE EFFECTS OF OUTBOARD MOTOR EXHAUSTS AND SELECTED AROMATIC TOXICANTS ON FISH

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Abstract—A continuous flow bioassay system was designed to measure the effects of outboard motor exhaust (OME) emissions and selected volatile and evaporative aromatic toxicants on goldfish (*Carassius auratus*). Continuous flow bioassays were run for 24, 48, 72, 96, and 720 h to determine lethal concentrations for 50% of individuals (LC-50's) for leaded OME, non-leaded OME, toluene, xylene, and 1,3,5 trimethylbenzene, the three individual compounds having been identified as significant aromatic components of OME. The 96 h LC-50's for these substances were found to be 171, 168, 23, 17, and 13 ppm, respectively. The values of 171 and 168 ppm for the two OME's are given in terms of gallons of fuel burned per million gallons of water. The continuous flow bioassay method was demonstrated to be a more reliable indicator of the effects of OME pollutants on aquatic organisms than is the static bioassay method.

INTRODUCTION

The utilization of the nation's waterways by boats powered by two-cycle outboard engines has increased significantly during the last decade. It is therefore important to inquire into possible stresses from outboard motor exhaust (OME) products on our aquatic environment. Since little research has been conducted on the effects of OME on aquatic organisms, it is not known whether or not OME affects aquatic animal or plant populations. One means of assessing both qualitatively and quantitatively the effects of potential pollutants on aquatic organisms is through the laboratory bioassay. Most bioassay studies with hydrocarbons reported in the literature have been done under static conditions (Turnbull, 1954; Wallen, 1957; Tagatz, 1961; and Pickering and Henderson, 1966). Since many of the hydrocarbons found in OME water are volatile, it is suggested that more reliable bioassay results may be obtained using a continuous flow system designed specifically for volatile hydrocarbons. However, none of the various types of continuous flow apparatus discussed in the literature (Alabaster and Abram, 1965; Betts, Beak and Wilson, 1967; Burke and Ferguson, 1968; Grenier, 1960; Henderson and Pickering, 1963; Jackson and Brungs, 1966; Lemke and Mount, 1963; Mount, 1962 and 1968; Mount and Brungs, 1967; Mount and Warner, 1965; and Solon, Lincer and Nair, 1968) are capable of handling highly volatile and evaporative hydrocarbons adequately because they are not designed to mix thoroughly and put slightly soluble hydrocarbons into solution or to prevent evaporation of volatile toxicants. Furthermore, none of the systems renews the volume in the test containers fast enough to keep pace with evaporation of toxic hydrocarbons. Therefore, a continuous flow bioassay system was designed to overcome these problems.

This continuous flow bioassay system was then used to determine LC-50's for goldfish (*Carassius auratus*) exposed to leaded OME water, non-leaded OME water, toluene, xylene, and 1,3,5 trimethylbenzene. The specific compounds used in this experiment were chosen for study because they are three of the aromatic components found in appreciable amounts in OME water. Weber, Cole and Posner (1974) have conducted studies of the components of exhaust emissions from a number of two cycle outboard engines of different size and manufacture, operated under a broad range of conditions. Gas phase emissions and condensable fractions of the exhaust were analyzed. The composition of the gas phase exhaust hydrocarbons resembled the composition of the fuel with the

principal exceptions that the olefin concentration was greater and the paraffin concentration slightly less than in the test fuel. A moderate variation in composition was evident from engine to engine. The condensable fraction was found to contain paraffinic, olefinic, and aromatic hydrocarbons as well as small amounts of phenols and carbonyl compounds. The composition of the condensate was very similar to that of the fuel. Aromatic compounds constituted 20–25% of the total condensed hydrocarbon amount. Toluene was slightly lower on a percentage basis in the condensate than in the fuel, and binuclear aromatics were slightly higher. According to Weber *et al.* (1974), the total amount of condensable material which can reasonably be expected to be condensed in a boating situation varies from about 1.5–7.0% of the fuel used.

EXPERIMENTAL DESIGN AND METHODS

The bioassay system designed for this study is depicted schematically in Fig. 1. As shown in Fig. 1, Ann Arbor tap water flows through two carbon filters 61 × 15 cm each (24 × 6 in.), which effectively dechlorinate the water by reducing active compounds of chlorine to chloride. The dechlorinated tap water moves through 1.77 cm polyvinyl chloride (PVC) pipe (schedule 80) to 2 208-l. (55 gal) drums. These drums are used to store either a stock solution of OME water or other test solutions of individual aromatic hydrocarbons. Stock solutions of OME water were made by collecting the OME products from either non-leaded gasoline (Indolene Clear) or leaded gasoline (Indolene 30, containing 0.79 g l^{-1} or 3 g gal^{-1} of tetraethyl lead) produced by two 2 HP Johnson outboard motors mounted in 2 208-l. (55 gal) drums. The amount of fuel run through the engines was monitored using 250 ml dispensing burets with stopcocks. The amount of water in the 208 l. (55 gal) drums was calibrated and controlled by overflow pipes. One drum held 200 l. (52.48 gal) of water, while the other held 207 l. (54.69 gal) of water. Therefore, the concentration of stock solution of the OME water can be calculated as gal of fuel burned per million gal of water or as parts per million (ppm). Exhaust fumes from the outboard motors which were not absorbed into the water were vented through exhaust hoods. Stock solutions of individual compounds were made by mixing a known quantity of compound in each 208 l. (55 gal) drum. These compounds, toluene, xylene, and 1,3,5 trimethylbenzene, were obtained from Mallinckrodt as analytical reagents (AR) assayed by the American Chemical Society (ACS). A homogenous solution of these compounds was obtained by vigorously mixing each drum simultaneously with a 1 2 h.p. motor (1725 rev min^{-1}) driving two stirrers via V-belts. Each drum is covered with a 3.18 mm (1.8 in.) piece of aluminum sealed with rubber weather stripping, and fastened down with four 1.77 cm (1.2 in.) eye bolts. As a consequence, there is very little evaporation of the stock solution from the drums. The drums interconnect, allowing flow between the identical stock solutions. A submersible pump is located in one drum and pumps the stock solution through a long glass (10 mm) tube into a wide mouth, 19 l. (5 gal) glass jug. The glass tube is sealed into the cover of the barrel by a neoprene rubber stopper. A liquid level controller (Dyna Sense Electronic, Model 7186) controls the levels of the stock solution entering the first jug from the drum. Jugs 2, 3, and 4 are filled from connections going from jugs 1, 2, and 3. Gravity flow-through glass tubing and 4 mm glass stopcocks controls the flow rate of the stock solution from the jugs to constantly stirred reactor chambers. The upper and lower probes from the liquid level controller are placed close together to minimize changes in flow rates and maintaining flow within ±2%.

The dechlorinated tap water was pH adjusted with CO_2 and distributed to the constantly stirred reactors from a constant head tank. The tap water was then sent through glass tubing and 4 mm glass stopcocks which control the flow rate. The flow rates from the stock solution and the dechlorinated tap water were calibrated to renew the volume in the 37.85 l. (10 gal) test chambers every 1 1/2 h. This renewal rate was sufficient to overcome evaporation of the volatile aromatic hydrocarbons and maintain homogeneous conditions. Fish receive doses of this homogeneous mixture from a standpipe in the reaction chamber. All reactor chambers are covered with 3.18 mm (1/8 in.) aluminum sheets. The glass tubes going through this sheet are inserted through neoprene stoppers, and the rod from the stirrer blade goes through a teflon bushing to the 1550 rev min^{-1} mixer. A rubber seal between the top edge of the reaction chamber and the 3.18 mm (1/8 in.) aluminum sheet with a 11.34 kg (25 lb) weight on it makes a tight seal. Water from the test chambers was eliminated through standpipes. Artificial lighting is provided by a row of 40 W fluorescent lamps 61 cm (24 in.) above the water surface. These lamps are controlled on a 24 h cycle by a timing device which is adjusted to approximate the natural photoperiod of light and darkness during the sea-

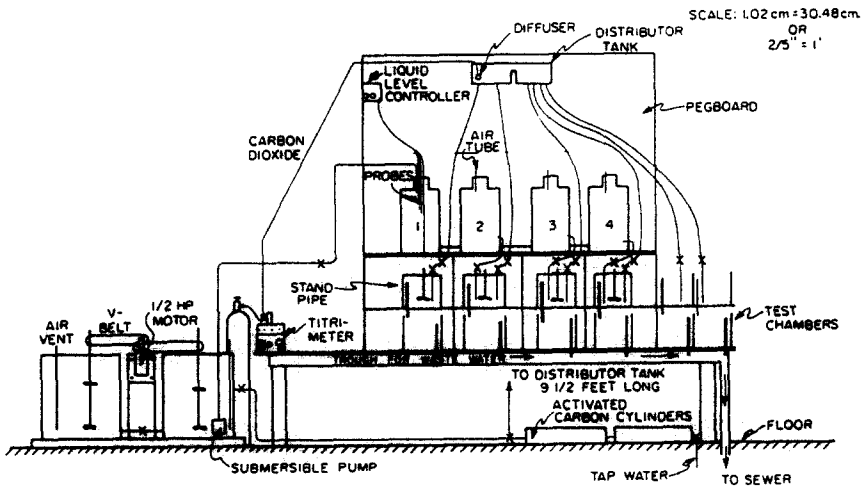

Fig. 1. Schematic diagram of the experimental apparatus.

Table 1. Ann Arbor tap water analysis

Methyl orange alkalinity	34 ppm as $CaCO_3$
Phenolphthaline alkalinity	37 ppm as $CaCO_3$
Total hardness	80 ppm as $CaCO_3$
pH	10.0
Chlorine	0.5 - 0.7 ppm
Calcium	21.6 ppm
Magnesium	5.3 ppm
Chromium	< 0.002 ppm
SiO_3	8 ppm

son that the bioassay was run. Natural seasonal variations in ambient temperatures were used for these experiments. Temperatures in the tanks varied from a low of 10°C in winter to 27°C during the summer months.

The efficiency of this continuous flow bioassay system was tested by determining 24, 48, 72, 96, and 720 h LC-50's for goldfish exposed to leaded OME, non-leaded OME, toluene, xylene, and 1,3,5 trimethylbenzene.

The basic procedure for the leaded and non-leaded OME emission experiments consisted of preparing a stock solution of OME water by running a known concentration of fuel at the recommended 50:1 fuel:oil ratio through a Johnson 2 h.p. engine at full throttle. After the stock solution was prepared, the engine was removed from the 208 l. tank and the solution tank covered immediately with a 3.18 mm thick aluminum cover to prevent evaporation of exhaust components from the water. Great care was taken not to exceed the solubility limits of OME in water by monitoring the u.v. absorbance of the test solutions. Therefore, a homogeneous solution could be obtained. Further, the stock solution was renewed every 12–16 h. The dechlorinated tap water analysis is given in Table 1. Adjustment of pH was accomplished by bubbling gaseous CO_2 into tap water using a Fisher automatic titrimeter (Model 36). The pH in the test chambers was so maintained at 7.0 ± 0.3. Water temperature varied from 17–19°C throughout the bioassay tests because they were run during the late winter and early spring. Dissolved oxygen (DO) was checked initially using the azide modification of the iodometric method (modified Winkler) as described in *Standard Methods* (1965). However, the DO was always 7 ppm or higher because of a rapid turnover of water. Flow rates were frequently checked, and u.v. analysis (Beckman DU) was used to monitor the system at all times. The outboard motor exhaust water was monitored as ppm of toluene at 210 nm. The exact procedure was used for the toluene, xylene, and 1,3,5 trimethylbenzene experiments, except that the stock solutions were made in ppm by mixing the aromatic compounds and water vigorously with two large mechanical stirrers for 10 min (see page 166 and Fig. 1). Concentrations of the three aromatic compounds tested were monitored with a Beckman DU spectrophotometer at 210 nm.

Each LC-50 was determined by exposing six fish per aquarium to three different concentrations of the OME test compounds. One control aquarium with six fish was run with each LC-50 test. Fish exposed for 720 h were fed a diet of Trout Chow Developer. All dead dish were immediately removed from their respective containers. Duplicate runs on the LC-50's were made to check for reliability of results. Deaths of goldfish from the range of concentrations were recorded from 24 to 720 h so that dose-response curves could be determined by a computer program (Daum, 1969; Finney, 1952).

All fish for the bioassay experiments, measuring 13–20 cm (5–8 in.) in length and weighing 20–80 g, were obtained from Grassy Fork Fisheries in Martinsville, Indiana, where they were raised from breeding stock in ponds. These fish, varying in age from 1–1½ years old, were fed a diet of 6–10 pellets of Purina Trout Chow Developer per day. All experimental and control goldfish were maintained in two fiberglass stock tanks containing 738 l. (195 gal) and 2725 l. (720 gal) of dechlorinated and pH controlled water that passed through the tanks at a flow rate of 3.79–7.57 l. (1–2 gal) min^{-1}. *Standard Methods* (1965) was used as a guideline for the preparation of fish before experimentation.

RESULTS

The compounds tested, fish used, and a summary of the results are shown in Table 2. The 24, 48, 72, 96, and 720 h LC-50 values and 95 percent confidence limits are reported as ppm of compound added to the test solution. All of the bioassays were run using

Table 2. Summary of median tolerance limits (ppm) for goldfish exposed to hydrocarbons from outboard motor exhaust (OME) emissions

Compound	24-hr. LC-50	Conf. limits	48-hr LC-50	Conf. limits	72-hr LC-50	Conf. limits	96-hr LC-50	Conf. limits
Leaded (OME)	226.15	199.89-285.87	203.24	183.87-248.92	191.67	174.36-225.69	170.68	156.33-186.84
Non-leaded (OME) water	211.92	188.11-262.31	193.68	174.50-230.43	183.05	166.49-216.51	167.87	145.88-181.05
Toluene AR (ACS)	41.59	31.99-71.72	27.62	21.58-36.01	25.33	20.13-31.85	22.80	17.07-29.95
Xylene AR AR (ACS)	30.55	26.42-37.26	25.10	19.16-31.01	20.72	12.04-24.95	16.94	6.85-21.31
1,3,5 TMB AR (ACS)	20.57	17.72-25.26	16.17	13.85-18.71	13.65	11.21-16.15	12.52	9.89-15.05

	720-hr LC-50	Conf. limits limits
Leaded (OME) water	104.91	84.13-126.78
Toluene AR(ACS)	14.58	10.73-19.96

the specially designed continuous flow bioassay system discussed above. The results show that the 96 h LC–50 is lower than the 24 h LC–50 for all compounds tested. The highest fish mortality occurred over the first 24 h. Of the three aromatic compounds tested, 1,3,5 trimethylbenzene is the most toxic, followed by xylene and toluene. Although there are numerical differences in the results for these three compounds, the differences are relatively small, and the compounds can be considered quite similar in their toxic effects on fish. The LC–50's for the leaded OME water and non-leaded water are also quite similar. The non-leaded OME water are reported in ppm as converted from the ratio of l. of fuel consumed to l. of dilution water. Finally, the results from the 30 day test also show that the 720 h LC–50 is lower than the 96 h LC–50 for all compounds tested. All control fish survived the time duration of each bioassay test.

DISCUSSION

The determination of 24, 48, 72, and 96 h LC–50's for leaded OME water, non-leaded OME water, toluene, xylene, and 1,3,5 trimethylbenzene reveal that goldfish can withstand relatively high concentrations of these compounds. Other researchers have reported even higher concentrations, largely because they used static tests instead of continuous flow tests for their experiments and lost most of the original concentration by evaporation during the first 24 h. For example, Pickering and Henderson (1966) reported 96 h LC–50 values for toluene and xylene of 59 and 35 ppm respectively, while this study reported 23 and 17 ppm for these same compounds (Table 2). Further, the results of Wallen, Greer and Lasater (1957) appear as if they used supersaturated systems to get their dilutions because they reported a 24 h LC–50 for toluene in mosquito fish (*Gambusia affinis*) of approximately 1000 ppm. They also stated that all fish appeared normal at 560 ppm and below. McKee (1963) reports that toluene has a solubility of only 470 ppm in water at 16°C. Although Wallen *et al.* (1957) used a different species of fish than those used by Pickering and Henderson (1966) and those used in this study, there should not be a difference of this magnitude in LC–50 values. It is suggested that all LC–50 data with highly volatile compounds, such as aromatic hydrocarbons, be reexamined using continuous flow bioassays.

Another important difference between the results of this study and those of other researchers is that the 96 h LC–50's are lower than the 24 h LC–50's for all the compounds tested. In contrast, Pickering and Henderson (1966) did not find any lower values for their 96 h tests as compared to their 24 h tests for toluene and xylene with goldfish. The reason for this also appears to be due to the use of static test systems, so that they may have lost most of their original material by evaporation, probably before the first 6 h of the LC–50 test. English, McDermott and Henderson (1963) have shown that there is a decrease in acute toxicity of OME water upon aging, which may also be ascribed to evaporation.

The continuous flow bioassay system that was designed for this study has the capability of fast renewal of the compound being tested, good mixing of these compounds to achieve homogeneous conditions, and minimal evaporation of the compound being tested because of enclosure of the system. The bioassay system is capable of running continuously for an unlimited period of time with minor maintenance of equipment. The major problem with the experimental unit used for this study was that stock solutions had to be renewed every 12–16 h. This problem can be circumvented by using larger stock tanks. Metering pumps would provide a more uniform flow rate of the stock solution. The system as described is reliable and does give reproducible results. The basic design is sound and can be easily modified to include larger storage tanks and test solution metering pumps.

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