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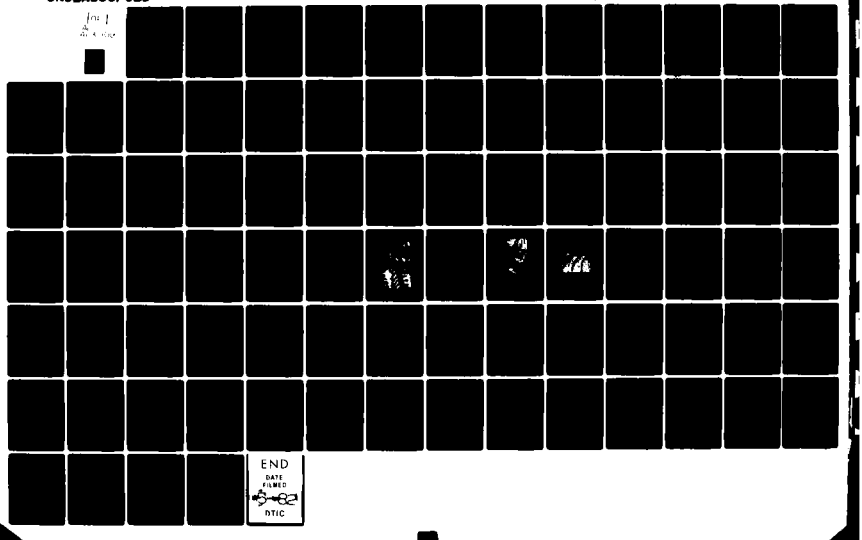

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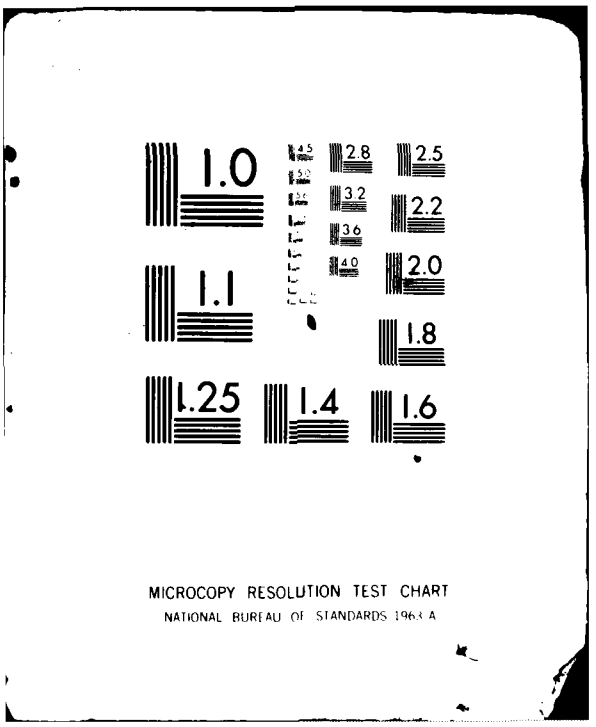
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Annual Technical Report
November 1981

IDENTIFICATION AND QUANTIFICATION OF THE WATER SOLUBLE COMPONENTS OF JP-4 AND A DETERMINATION OF THEIR BIOLOGICAL EFFECTS UPON SELECTED FRESHWATER ORGANISMS

NORTH DAKOTA STATE UNIVERSITY
FARGO, ND 58105

Drs. Brammer and Puyear

Controlling Office: USAF Office of Scientific Research/NL
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A paper describing the use of C-18 Sep-Pak mini-columns for concentrating JP-4 jet fuel alkylbenzenes is included, and has been accepted for publication.

The solubility of JP-4 alkylbenzenes in water of four salinities and at five different temperatures have been measured. The LC 50 and MATC for toluene on fathead minnow embryos, 1-day posthatch protolarvae and 30-day old fish has been determined.

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A detailed embryonic developmental sequence for the fathead minnow at 25 C for the first 49 hours has been described.

Benzene metabolism by 12,000 x g liver supernatant was measured in a closed vial system using a G.C.

Scanning electron microscopy was used to examine the gill lamellae of control and toluene exposed fathead minnows.

WORK SCOPE(SEPTEMBER 30,1980-SEPTEMBER 29,1981)

This phase of the research entailed:

I. Completing the work relating to the use of C-18 Sep-Pak mini columns for concentrating water-soluble JP-4 derived alkylbenzenes. This research has been submitted and accepted for publication in the BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY. This paper will appear in vol. 27 no. 6 (December), 1981.

II. Determining the solubilities of the major JP-4 jet fuel alkylbenzenes in water at five different temperatures and four different salinities. This study has been completed and a manuscript is in final stages of preparation.

III. Completing studies to determine the LC 50 and MATC for toluene in fathead minnow embryos, 1-day posthatch protolarvae and 30-day old fish. This study is the basis of a manuscript which has been completed and is included as a part of this report. It will be submitted to BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY.

IV. Preparing a manuscript describing the water-soluble Lethal and No-Effect Levels of Toluene on the Fathead Minnow. Analysis of these exhausts was completed using techniques and equipment described under phases I and II above. This manuscript has been accepted for publication in ARCHIVES FOR ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY, and is included as Appendix B of this report.

V. Continuing with the description of early stages of embryonic development in the fathead minnow at 20 C and 25 C. The effects of toluene exposure upon development are also under investigation.

VI. Continuing the measurement of benzene and toluene metabolism by the 12,000 x g liver supernatant.

VII. Initiating scanning electron microscopy (SEM) studies on control and toluene-exposed fathead minnows.

I. A copy of the accepted manuscript: USE OF REVERSE PHASE C-18 MINI-COLUMNS FOR CONCENTRATING WATER-SOLUBLE HYDROCARBONS submitted to BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY is included in Appendix A.

II. The solubility of the major JP-4 jet fuel alkylbenzenes was determined using the following parameters:

A. Five water temperatures over the range of 3C and 37C.

B. Four different water salinities for each of the five temperatures chosen. These specific temperatures and salinities were chosen because they encompass the range in which most freshwater aquatic organisms are found.

The manuscript in which the results of this study are described is given below.

SOLUBILITY OF MAJOR JP-4 JET FUEL ALKYLBENZENES IN WATER OF DIFFERENT SALINITY AND TEMPERATURE

R.L. Puyear, K.J. Fleckenstein and J.D. Brammer

Introduction

JP-4 jet fuel is primarily kerosene, although actual composition varies considerably from one batch to another. Kerosene consists of approximately 40% alkanes (by weight) and 2% alkylbenzenes (SNYDER, et al. 1963). MORROW (1974) has shown that the alkanes are relatively harmless in an aqueous environment. The alkylbenzenes, however, are far more toxic. JP-4 used in this study included approximately 4% toluene (by volume) and 2-3% xylenes (m-, p-, and o-xylene).

PICKERING, et al. (1966) found varying degrees of toxicity of some alkylbenzenes depending upon water hardness, pH, and temperature. Other studies have shown that any one or combination of these factors could influence the water-solubility of various hydrocarbons (BOBRA, et al., 1979; FRIANT and SUFFET, 1979). BOBRA, et al., (1979) reported the solubility of aromatic hydrocarbons was less in sea water than fresh water. MCAULLIFE (1971) found the solubility of aromatic hydrocarbons such as toluene and benzene decreased as the concentration of NaCl increased. FRIANT and SUFFET (1979) demonstrated that although temperature and electrolyte concentration influenced the solubility of the hydrocarbons they studied, pH had no significant influence upon solubility. In this study we examined the effects of water hardness and temperature upon the solubility of hydrocarbons in JP-4.

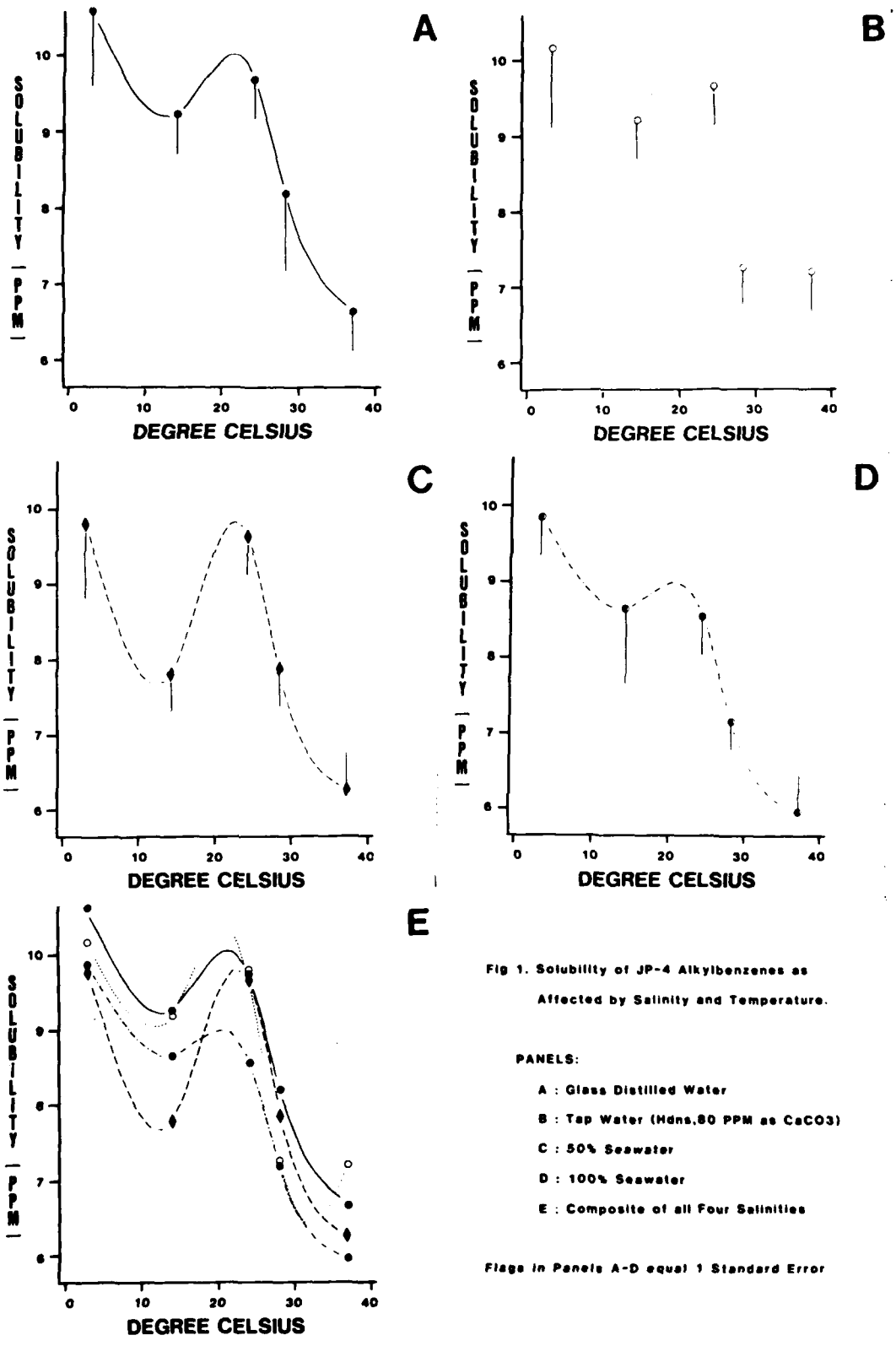

Fig 1. Solubility of JP-4 Alkylbenzenes as Affected by Salinity and Temperature.

PANELS:

A : Glass Distilled Water

B : Tap Water (Hdns.80 PPM as CaCO3)

C : 50% Seawater

D : 100% Seawater

E : Composite of all Four Salinities

Flags in Panels A-D equal 1 Standard Error

Materials and Methods

The hydrocarbon standards used were analytical grade (Alltech Assoc.). Solvents were spectral grade (Burdick and Jackson). The JP-4 jet fuel was obtained from the North Dakota Air National Guard, Fargo, N.D. Hydrocarbons were concentrated using mini-columns (Sep-Pak C-18, Waters and Assoc.) according to procedures described previously (PUYEAR et al, in press).

Fargo tapwater has a hardness of 80 PPM $CaCO_3$. Seawater was prepared using Instant Ocean (Aquarium Systems Inc., Wickliffe, OH) synthetic sea salts in tap-water. Full-strength seawater had a specific gravity of 1.025; specific gravity of half-strength sea water was 1.015; $CaCO_3$ hardness was 6400 and 3200 PPM respectively.

Water-soluble fractions of JP-4 were prepared by slowly mixing 100 ul of JP-4 in 500 ml hydrocarbon-free water for 2 hours in silanized 1 liter glass stoppered erlenmeyer flasks. This volume provided concentrations of individual water-soluble components which could readily be quantified, but which were still below their reported saturation level in water. This solution was then put into a separatory funnel and allowed to stand for 2 hours. Following this, 200 ml of the JP-4 water mixture was drawn from the separatory funnel and concentrated using a C-18 Sep-Pak (PUYEAR et al, in press). In those cases where the vapor phase was sampled, mixing was done in a 24/40 standard tapered 1 liter

erlenmeyer flask fitted with a 24/40 standard tapered syringe needle adaptor stopper. After mixing for two hours, the vapor phase in the flask was analyzed chromatographically. Samples were injected using a Valco 6-port valve maintained at room temperature. This injection valve was fitted with a 250 ul sample loop. The sample loop was filled by evacuating it to a negative 15 pounds per square inch. After duplicate chromatographic analyses of the headspaces, the aqueous phase was then poured into a separatory flask and processed as described above.

Hydrocarbons were detected using a Varian 3760 gas chromatograph with a flame ionization detector. A glass chromatographic column (2 mm i.d. by 1.8 m) was packed with 10% 1,2,3-tris (2-cyanoethoxy) propane (TCEP) on 100/200 mesh Chromosorb PAW (Supelco, Inc.).

Components of JP-4 were identified by comparison with retention times of standard hydrocarbons and by peak-enhancement methods. The aliphatics were not baseline-resolved, and could not be conclusively identified. Concentrations of alkylbenzenes were calculated using the external standard method, with a Spectra Physics SP-4000 data system. Concentrations are reported in PPM by volume (ul/l). The data analysis was performed using Statistical Analysis System (SAS, 1979), and Duncan's Multiple Range was employed for testing significant differences in alkylbenzene solubilities. SAS/GRAPH(1981) was used for plotting data.

Results

The eight alkylbenzenes present in highest concentration in JP-4 that have been identified and quantified are listed in Table 1 These concentrations were obtained by chromatographic analysis of JP-4 in ethyl acetate. The aliphatics appear in much higher concentrations in JP-4, but because of their low water-solubility and high volatility, they were not present in significant (nor reproducible) amounts in the water-soluble portion.

TABLE 1

PERCENT OF MAJOR ALKYLBENZENES IN JP-4

COMPONENT	PERCENT
Toluene	7.3
Ethylbenzene	0.6
Xylenes(m & p)	2.3
o-xylene	1.0
3,4-ethyltoluene	0.7
2-ethyltoluene	0.2
1,2,4-trimethylbenzene	1.2
1,2,3-trimethylbenzene	0.1

Benzene was not detected using the present chromatographic conditions. JP-4 was added to ethyl acetate (2 PPM v/v) and 1 ul chromatographed.

Maximum aqueous solubility of JP-4 alkylbenzenes are listed in Table 2 These were obtained by mixing JP-4 in glass-distilled water at 25 C for 2 hours; then placed in a

separatory funnel for two hours before concentrating with a Sep-Pak. At lower temperatures these values should increase somewhat. It should be noted here that, even in the highest concentrations, the maximum solubility for any single compound was 30 PPM or less.

TABLE 2

MAXIMUM CONCENTRATION OF JP-4 DERIVED ALKYLBENZENES IN GLASS-DISTILLED WATER AT ROOM TEMPERATURE

COMPONENT	CONCENTRATION
Toluene	30
Ethylbenzene	3
Xylenes(m & p)	6
o-xylene	2
3,4-ethyltoluene	1
2-ethyltoluene	0.3
1,2,4-trimethylbenzene	1
1,2,3-trimethylbenzene	0.5

Each value is the mean of 4 duplicate determinations.
Values are expressed as PPM(v/v).

With a view to investigating possible antagonistic effects of alkylbenzenes, the experiment summarized in Table 3 was performed. Starting with toluene in glass-distilled water, and progressing on to a mixture of 4 compounds, single hydrocarbons were mixed in glass-distilled water for 2 hours and concentrated with a Sep-Pak as described above. The volumes of hydrocarbon added in these samples were comparable to the volumes in JP-4 at saturation level.

TABLE 3

RECOVERY OF ALKYLBENZENES FROM GLASS-DISTILLED WATER WHEN ADDED IN VARIOUS COMBINATIONS

	COMPONENTS RECOVERED (PPM)				
COMPONENTS	Tol	Xyl	O-xyl	Et-benz	Total
Tol	213				213
Tol+Xyl+O-xyl	106	34	37		177
Tol+Xyl+O-xyl +Et-benz	98	24	43	30	195

Each value is the mean of 4 duplicate determinations. Values are expressed as PPM(v/v). Abbreviations: Tol, Toluene; Xyl, m & p-xylene; O-xyl, o-xylene; Et-benz, Ethylbenzene.

Results indicate the amount of toluene recovered in the aqueous phase, when it was the only hydrocarbon present, was greater than when it was present with other compounds. This may account for the low solubilities for these compounds when present in JP-4. It can also be seen that the total alkylbenzenes recovered in each of the test parameters studied was about the same.

The compounds making up JP-4 have, in general, very low water solubilities. In order to establish what proportion of the JP-4 went into the vapor phase, samples were mixed in glass stoppered erlenmeyer flasks with stoppers that allowed the vapor phase to be sampled and analyzed chromatographi-

cally before the aqueous phase was concentrated. It was found that even at low concentrations of JP-4 (100 PPM or approximately 10 PPM alkylbenzenes) as much as 50% of the toluene could not be accounted for in either the liquid or vapor phases. Even at these low concentrations, an "oil slick" layer was observed between the aqueous and vapor phase . It was concluded that the alkylbenzenes not accounted for were found in this interface. When larger volumes of JP-4 were added to the surface of the aqueous phase in the flask, it did not appreciably increase the concentration of alkylbenzenes in either the headspace or aqueous phases. The amount in the "oil slick" simply increases.

The effects of temperature and water hardness on solubility were investigated using glass-distilled water, tap water, 50% seawater, and seawater. The temperatures used were 3, 14, 24, 28, and 37 C. Although the graph (Figure 1) shows a dip in solubility between 0 C and 28 C, this is not statistically significant. The unusual shape of the curves are probably due to the non-ideal nature of the system.

Discussion

The parameters chosen for water quality in the solubility tests were used to represent the range of conditions under which aquatic organisms can normally live. The most significant effect was due to temperature. The maximum solubility occurs at a temperature just above freezing, with a second maximum at about 28 C.

Solubility values for toluene and the xylenes (Table 2) are considerably below the solubilities reported by MCAULLIFE (1966, 1971). His work was done with single compounds, and not with complex mixtures such as JP-4. This may account for the reduced aqueous solubility. This was further substantiated by the data reported in Table 3. The total alkylbenzene solubility was about equal, no matter whether a single, or several components were present in the aqueous system.

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III. The Lethal Concentration for a 50% kill (LC 50) and a Maximum Acceptable Toxic Concentration (MATC) for toluene was determined for the following developmental stages of the fathead minnow:

A. Embryo

B. 1-day posthatch protolarvae

C. 30-day old fish

A text of the manuscript and results follow:

LETHAL AND NO-EFFECT LEVELS OF TOLUENE ON THE FATHEAD MINNOW,

PIMEPHALES PROMELAS.

E.W. Devlin, J.D. Brammer, and R.L. Puyear

Introduction

Toluene is an aromatic hydrocarbon widely used as an industrial solvent. It is also a major component of the water soluble fraction of refined oils (Puyear, et al., 1981; Wolfe, 1977). Like other aromatic hydrocarbons, toluene is

toxic to many aquatic organisms (Berry and Brammer, 1977; Malins, 1977; Dunstan, 1975; Potera, 1975; Tatem, 1975).

Most toxicity tests with toluene have been performed under static conditions on post-embryonic aquatic organisms. Little work has been done on toluene's effect on embryonic and early larval stages, although these stages are known to be more sensitive to toxic stress (Stoss and Haines, 1979; Ernst and Neff, 1977). Early life cycle testing is helpful in evaluating toxic effects of a compound.

The LC 50 value (lethal concentration to 50% of the population) is a commonly used index of a compound's toxicity, while the Maximum Acceptable Toxic Concentration (MATC) is often used as a criterion for determining "safe" levels of a compound in the environment (Mount and Stephan, 1967). This study was designed to determine both the LC 50 and MATC values of toluene on the fathead minnow, Pimephales promelas. The 96-hour LC 50 values were determined for embryos, 1-day posthatch protolarvae (Snyder et al., 1977) and 30-day old fish. The MATC value was determined for 30-day old minnows.

Methods

Several bioassay systems have been designed to provide continuous or intermittent supply of control water and water water-toxicant mixtures to a series of exposure tanks (Lemke, et al., 1978; Peltier, 1978; Defoe, 1975; Brungs and Mount, 1970). These systems typically are larger than necessary for toxicity tests with fish embryos and larvae. A

small enclosed mini-diluter system described by Benoit-a, et al. (in press) and a larger multichannel toxicant injector diluter system described by Defoe (1975) were used as exposure systems during this study.

The Defoe diluter system was used for a 96-hour LC 50 test with 30-day old fathead minnows, and a 32-day embryo larval test. Twelve 5 liter glass exposure tanks were fitted with stand-pipes to maintain a water depth of 5 inches. The diluter board was set to deliver a flow rate of 1 liter every 5 minutes to each exposure tank. This minimized the problem of volatilization of the toluene, and maintained constant dissolved oxygen levels. Four ounce glass jars fitted with stainless steel screen bottoms served as egg cups during the embryo-larval test. Egg cups were suspended from a motorized rocker arm assembly. The gentle up and down motion provided constant exchange of water in the egg cups. The entire system was enclosed in clear plastic, and maintained under negative air pressure. Filtered Lake Superior water (pH 7.6, hardness 45 PPM eq. CaCO3) maintained at 25 C was used as dilution water. The 96-hour LC 50 tests of toluene on fathead minnows were performed following procedures described by the US EPA (1975). A 32-day embryo-larval test was conducted to determine the no effect concentration of toluene on fathead minnow weight. Embryo-larval test procedures followed guidelines outlined by McKim (1977). Toluene concentrations were determined with a

Baird-Automic model SP100 ratio recording spectrofluorimeter.

The mini-diluter system described by Benoit, et al., (1981) was used to run 96-hour LC 50 tests on embryos, 1-day old protolarvae, and 30-day old fish. Carbon filtered Fargo tap water (pH 8.3, hardness 80 PPM eq. CaCO3) was used as dilution water. Test procedures followed those recommended by US EPA (1975). Toluene concentrations in the exposure tanks were determined using Sep-Pak C-18 and a Varian GC 3700 (Puyear, et al., in press). The 96-hour LC 50 values and their confidence limits were calculated using an interactive computer program developed by Hanes, et al., (1980). This program essentially follows the probit analysis described by Finney (1971), except it does not require partial kills. Values were considered significantly different when the 90% confidence limits about the LC 50's did not overlap (American Public Health Association, 1976).

Results

The 96-hour LC 50 values for the three age groups of fathead minnows used in this study are given in Table 4. The range of 96-hour LC 50 values for embryos is 55-72 mg/l. Although this range appears large, these values are not significantly different (alpha=0.10). The 96-hour LC 50 values for 1-day posthatch protolarvae have a smaller range (25-36 mg/l), and again these differences are not significant (alpha=0.10). The 96-hour LC 50 values for 30-day old fathead

minnow obtained from the mini-diluter system are 26, 30, and 31 mg/L. None of these values are significantly different (alpha=0.10). The results of the 96-hour LC 50 test with 30-day olds performed with the Defoe diluter gave a value of 18 mg/l, which is significantly different (alpha=0.10) from the other three values for 30-day olds.

TABLE 4
96 HOUR LC 50 VALUES OF TOLUENE ON FATHEAD MINNOWS

	30-DAY OLDS	LARVAE	EMBRYOS
TEST	LC 50	LC 50	LC 50
1	30 (23-42) mg/l	36 (29-44) mg/l	72 (55-107) mg/l
2	31 (24-44) mg/l	25 (21-29) mg/l	66 (56-78) mg/l
3	26 (26-33) mg/l	27 (23-32) mg/l	59 (51-68) mg/l
4	18 (16-20) mg/l	28 (21-34) mg/l	55 (46-66) mg/l

Replicate 96-hour LC 50 values of toluene for each of the three age groups of fathead minnows and their 90% confidence limits.

There is no significant difference (alpha=0.10) between the 96-hour LC 50 values obtained with the mini-diluter for the protolarvae and the 30-day old fish. However, values for both the protolarvae and the 30-day old fish differ significantly (alpha=0.10) from the LC 50 values for embryos.

Mean fish weight for each exposure concentration was used as an index of larval growth in the embryo-larval test.

Based on mean weight, the estimated MATC of toluene on 30-day old fathead minnows resulting from this test was found to lie between 0 and 4 mg/l.

Discussion

A number of 96-hour LC 50 values for toluene of freshwater teleosts have been reported: 54 mg/l for the Japanese medaka, Oryzias latipes (Stoss and Haines, 1979), 59 mg/l for the guppy, Poecilia reticulatus (Pickering and Henderson, 1966), 42 mg/l for the fathead minnow (Pickering and Henderson, 1966), 23 mg/l with goldfish, Cauassius auratus (Brenniman, et al., 1976), and 13 mg/l for the bluegill, Lepomis macochirus (US EPA, 1978). The LC 50 values reported in this study are similar to those reported by other researchers.

It is interesting to note that toxicity tests performed on 30-day old fish in this study following very similar procedures gave significantly different results. The variation in results is thought to be due to differences in diluter systems, water quality and differing toluene quantification procedures. The toxicity test that gave a value of 18 mg/l was performed with a Defoe diluter system in the US EPA laboratory, Duluth, Minnesota. The other three tests (30, 31, 26 mg/l) were done with the mini-diluter system at North Dakota State University.

Also of interest is the significantly greater resistance of embryos to toluene's toxic effects when compared with

protolarvae or 30-day old fish. Greater variation in the 96-hour LC 50 values for embryos may result from stress on the recently water-hardened embryos at the onset of the test. Embryos were exposed to toluene 1-2 hours following fertilization. It is not unusual for embryos to be more resistant to toxicant stress than larval or adult forms (Johnson and Julin., 1980). This resistance may be due to a lower metabolic rate of the embryos compared with newly hatched protolarvae or 30-day old fish. It may also be the result of toluene being sequestered in the lipid-rich yolk, making it unavailable for metabolism.

Growth of fish larvae is a sensitive indicator of toxic stress. Differential growth rates of larvae can have a marked effect on an organism's ability to compete in the ecosystem (Benoit-b et al., 1981). The 32- day embryo-larval test gave a MATC value of less than 4 mg/l based on mean weight. Studies are necessary to quantify the levels of toluene found in the environment to put the MATC and LC 50 values calculated in this and other studies in perspective. Further work is also necessary to characterize sublethal effects of aromatic hydrocarbons such as toluene on teleostean development.

Acknowledgments

This research was supported by Air Force Research Grant AFOSR-78-3709. A special thanks is given to Dr. Richard Seifert and colleagues at the US EPA Environmental Research

Laboratory, Duluth, Minnesota, who provided both expertise and physical facilities during the summer of 1979.

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IV. Water-soluble components of outboard motor exhausts was determined.

While the water-soluble components of JP-4 were under investigation, a separate comparison study was conducted to determine the water- soluble components of outboard motor exhausts. This study utilized equipment purchased by the USAF, as well as some of the expertise developed in the USAF studies. A manuscript reporting the results of this comparison study is attached to this report as Appendix B. It has been accepted for publication in the ARCHIVES FOR ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY. The contributions made by the USAF have been acknowledged.

V. A detailed investigation of fathead minnow development from fertilization through 49 hours at 25 C under normal conditions and after toluene exposure was continued from last year. The results of this investigation are summarized below:

Histological Studies

Egg production in the fathead minnow breeding system described in last year's report is at a maximum during the summer months. A series of toxicity tests were designed and performed during the summer of 1981 to take advantage of the high egg production. All toxicity tests were performed at

23.5 +/-0.5 C in the mini-diluter system described in last year's report. Embryos were then fixed in either FAA or Smith's solution. Embryos were dechorionated, washed, dehydrated and embedded in periplast for serial sectioning. A minimum of ten toluene treated and ten control embryos were embedded at each developmental stage tested (Table 5). One group of toluene treated and control embryos were embedded in epon for TEM and light microscopic examination.

TABLE 5. EXPOSURE CONCENTRATIONS OF TOLUENE AND AGES OF EMBRYOS FIXED IN SMITH'S SOLUTION OR FFA, AT ENDS OF TOXICITY TESTS.

						SMITH'S SOLUTION						
Embryo age (hours) at test end	4	7	23	25	41	43	48	49	52	60	75	124
Toluene conc. (mg/l)	60	60	45	45	45	100	45	45	100	50	40	40
						FAA						
Embryo age (hours) at test end			15		30		45		60		143	
Toluene Conc. (mg/l)			40		21		20		50		37,84	

The different age groups of embryos (toluene treated and controls) are currently being sectioned at 8um and stained with Harris Hematoxylin and Mallories Triple Connective Tissue stains. Methods are being developed to quantify to-

luene's effect on embryonic development in the fathead minnow.

Embryonic Development Sequence

Our studies have shown that excessive handling or exposure to bright lights result in increased activity of the fathead minnow embryos. This increased activity has also been noted by other researchers (McKim, 1981). Increased embryonic activity often results in a premature hatch and gives highly variable rates of embryonic development. A system was designed to observe developing embryos that minimizes the effects of handling and bright lights (Figure 2). This system consists of an elevated platform located inside an 8 inch by 7 inch by 12 inch glass tank. A partially submerged embryo chamber with fine nylon mesh bottom is located on the elevated platform. A mirror is located under the platform to reflect incident light into the embryo chamber. The glass tank containing the egg chamber is located in a constant temperature water bath. Aeration is provided to both the glass tank and surrounding constant temperature bath.

Eggs are removed from breeding tiles as described in previous reports, and placed into the embryo chambers. This is the only mechanical disturbance of the embryos. Binocular dissecting microscopes mounted on adjustable arms are located directly over the embryo chambers. The embryos are observed during development using incident room light only.

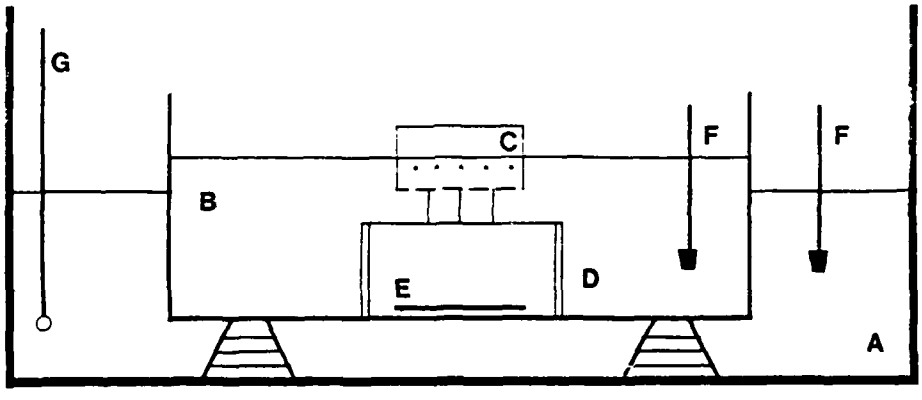

Figure 2. SYSTEM USED FOR DETERMINING DEVELOPMENTAL SEQUENCE OF FATHEAD MINNOWS. A) CONSTANT TEMPERATURE WATER BATH, B) GLASS TANK, C) EMBRYO CHAMBER, D) ELEVATED PLATFORM, E) MIRROR, F) AIR STONES, G) THERMOMETER.

Using this system, hatching occurs in 100 hours at 25 C and in 174 hours at 20 C following fertilization.

The sequence of embryonic development at 20 C has also been determined with this system for the first 49 hours of development. Below is a description of this sequence:

0.75 hours. 1-blastomere.

1.33 hours. 2-blastomeres.

1.66 hours. 4-blastomeres.

2.0 hours. 8-blastomeres.

2.33 hours. 16-blastomeres.

2.75 hours. 32-blastomeres.

3.25 hours. 64-blastomeres. Outermost blastomeres appear to be arranged in a more orderly fashion than those in the interior.

3.75 hours. Individual blastomeres of the blastoderm can no longer be counted because they are decreasing in size.

5.25 hours. Cleavage continues. Blastomeres are becoming smaller.

6.75 hours. High blastula. The blastula is seen as an elevated cap on the yolk mass. The periblast is clearly visible extending down from the edge of the blastula covering a small region of the yolk mass.

7.25 hours. Blastula is flattening out over the yolk mass.

7.75 hours. Continued flattening of the blastula over the yolk mass.

8.75 hours. The individual blastomeres appear very small. Yolk has a granular appearance.

10.25 hours. Flat blastula.

11.00 hours. Start of epiboly. The blastoderm has migrated 1/3 of the way down the yolk mass. An extensive periblast is present.

11.75 hours. Epiboly continues. Blastoderm covers almost 1/2 of the yolk mass. The germ ring is visible as a slightly elevated edge of the advancing blastoderm.

12.50 hours. Epiboly continues. Blastoderm covers 1/2 of the yolk mass. A slight thickening has occured at one point on the germ ring in the region of the embryonic shield.

13.25 hours. Epiboly continues. Blastoderm covers 1/2-5/8 of the yolk mass. The germ ring is thickened in the region of the embryonic shield, and is more clearly visible as the elevated edge of the advancing blastoderm.

14.25 hours. Epiboly continues. Blastoderm covers 5/8 of yolk mass. Germ ring has become a wide band.

15.00 hours. Epiboly continues. Blastoderm covers 3/4 of the yolk mass. A very thin layer of blastomeres are present in front of the advancing germ ring.

16.25 hours. Epiboly continues. Blastoderm covers 4/5 of the yolk mass. Blastomeres are thinning out over most of the covered yolk mass, except in the germ ring and embryonic shield.

17.25 hours. Epiboly continues. Yolk plug is protruding slightly.

18.25 hours. Epiboly continues. Embryonic shield has become less distinct. Edges of the germ ring surrounding the yolk plug are rough.

19.25 hours. Epiboly continues. Germ ring has not yet closed. A definite caudal cluster of cells is present at the posterior end of the embryonic axis. A slight depression in the yolk mass is seen ventral to the caudal cluster.

20.25 hours. Epiboly continues. The germ ring has almost closed over the yolk plug. Onset of neurulation. A second cluster of cells can now be seen at the anterior end of the embryo. The anterior and caudal clusters are connected by a low, broad ridge of cells with a very slight medial depression.

21.00 hours. The last stage of epiboly. The yolk plug is very small, but has not been covered by the germ ring. Cells covering the yolk mass appear to be migrating toward the embryonic axis. The anterior cluster of cells is quite broad laterally.

21.5 hours. Yolk plug is completely covered by the germ ring. The optic anlage are present as two lateral enlargements at the anterior end of the embryo. Kupffer's anlage is present as a depression in the yolk mass just ventral to the caudal knob.

22.25 hours. Notochord is forming. Kupffer vesicle is forming.

22.75 hours. Further definition of the notochord and consolidation of cells into the embryonic axis.

23.25 hours. Further migration of cells toward the embryonic axis, although the embryo is still quite broad.

24.25 hours. The optic anlage are forming. Kupffer vesicle is still present. Notochord lies in a slight depression in the yolk mass connecting the caudal and anterior knobs.

24.50 hours. 1-2-somites. Somites are not clearly separated.

25.00 hours. 3-somites.

26.00 hours. 5-somites. Optic vesicle primordia are present as lateral lobes in the prosencephalon region of the brain.

26.5 hours. 6-7 somites. Somites extend laterally from the embryonic axis into the broad lateral plate mesoderm. The caudal cluster is becoming smaller. The prosencephalon, mesencephalon and rhombencephalon are faintly visible.

27.25 hours. 7-somites. The notochord is quite distinct. The optic vesicles and prosencephalon form an arrow shaped mass of cells.

29.25 hours. 10-somites. Invagination of the optic vesicles. This invagination appears as a small slit on the lateral surface of the optic vesicles. The three regions of the brain are more distinct. Kupffer vesicle is still present as a depression in the yolk mass ventral to the caudal

cluster. The pericardium is forming as a fluid filled cavity anterior and ventral to the prosencephalon.

30.25 hours. 10-11-somites. The notochord and somites are more clearly defined. The pericardium is migrating posteriorly and lies ventral and lateral to the optic vesicles.

31.25 hours. 12-somites. Optic cups are forming with the further invagination of the optic vesicles.

13-14-somites. The optic cups bulge laterally from the prosencephalon. The embryonic axis is becoming well elevated above the yolk mass. The pericardium is enlarging.

35.25 hours. 16-17-somites. Further development of the optic cups.

36.25 hours. Lens placode is visible as a thickening on the lateral surface of the eye cups.

36.75 hours. Neuromeres in the region of the rhombencephalon are quite distinct.

37.25 hours. Otic placodes are forming laterally in the posterior rhombencephalon. Kupffer vesicle has become smaller. The pericardium continues to migrate posteriorly and laterally, and is located under the prosencephalon and mesencephalon.

37.75 hours. 22-somites. Tail bud is lifting off the yolk mass. Otic placodes are present as lateral thickenings of the epidermis in the rhombencephalon region. Olfactory placodes are forming anterior to the eye cups.

38.25 hours. Distinct regions have formed within the mesencephalon. The tail bud has separated further from the yolk mass.

39.25 hours. There is a marked reduction in the yolk mass.

40.25 hours. Elongation of the tail bud.

42.75 hours. 26-somites. The first twitching movements of the embryo are seen in the tail bud region. The optic capsule has formed. Cells of the notochord are large and distinct. A vesicle on the ventral aspect of the tail bud has formed. A furrow has formed that splits the three regions of the brain along the medial axis. A transverse furrow separates the prosencephalon from the mesencephalon.

44.25 hours. Heart is not yet visible.

48.75 hours. 30-somites. Somites extend well into the tail bud. Embryos are quite active, exhibiting swimming movements within the chorion. The heart is visible: ventral-posterior to the eye cups. The heart beat is weak and irregular. No vessels or blood are visible; only a few cells are seen moving within the heart. The lens has formed.

VI. Rate of metabolism of toluene by 12,000 x g enzyme supernatant was determined using the following parameters:

A. Rat liver has been used because of it's availability, and because an extensive literature exists dealing with xenobiotic metabolism by this preparation. In the near future, fathead minnow liver will be used.

B. Preliminary studies on the binding of toluene to this microsomal preparation has been performed. A more complete study about binding will be made with C14 toluene after the liquid scintillation counter arrives.

Preliminary results of toluene metabolism by liver microsomal enzymes follows:

Introduction

The metabolic activity of hepatic microsomal enzymes was studied using the following substrates: aminopyrine, and toluene. A protein assay was used to better correlate enzyme activity. Rats were used in the initial tests for three reasons: (i) extensive research involving hepatic mixed-function oxidases (MFO) has already been done using laboratory rats, (ii) rat livers are large and have no gall bladder (contamination of liver microsomes by bile apparently deactivates MFO systems), and (iii) rats are relatively easy to maintain. The combination of these elements make the rat ideal for use in developing techniques required for metabolic studies.

Procedures developed using the rat were then applied to the fathead minnow (Pimephales promelas). Preliminary results were sketchy but indicated a modicum of MFO activity. The fathead was chosen to compliment developmental studies described elsewhere in the report.

A. Preparation of Hepatic Microsomes

Twenty-four hour food-deprived test organisms were guillotined or otherwise beheaded and exsanguinated. (In the case of P. promelas care must be taken not to rupture the gall bladder and thereby contaminate the abdominal cavity with bile.)

Immediately upon sacrifice the organisms were opened, and the livers removed to a beaker of 0.154M KCl/0.1M phosphate buffer, pH 7.4, chilled in an ice bath. Livers were carefully rinsed, dried of excess fluids, and weighed. A 1:10 homogenate (liver:KCl/PO4 buffer) was made using a Tekmar SDT homogenizer or a teflon and glass Potter-Elvehjem homogenizer. Livers were maintained in an ice-bath throughout to reduce denaturation of enzymatic proteins, especially during homogenization.

Homogenates were centrifuged at 0-4C and 12,000 x g for twenty minutes to remove whole cells, nuclei, mitochondria, and other cell fragments. Supernatants were saved on ice if they were to be used that day, or were immediately frozen if they were to be used the following day. Pellets were discarded.

B. Aniline Hydroxylase Assay Procedure

Each reaction mixture contained 1.0ml of 12,000 x g supernatant, 1.5ml of cofactor (0.372mg NADP, 2.60mg glucose-6-phosphate, 2.40mg MgCl2 in KCl/PO4 buffer), and 0.5ml of aniline stock solution (0.14ml aniline + 9.86ml phosphate buffer, pH 7.4). This resulted in a reaction mixture with a total volume of 3.0ml with the following concentrations of reagents: 33.3mg liver (wet weight)/ml, 0.167mM NADP, 3.33mM G-6-P, 25.2mM MgCl2, and 25.6mM aniline.

After the prescribed incubation time, at 37C for rat liver microsomes or 30C for fish liver microsomes, in a Dubnoff water bath, 120 oscillations per minute, reactions were killed with 1.0ml 20% (w/v) trichloroacetic acid (TCA). Samples were then centrifuged for five to ten minutes using a table-top centrifuge to settle the TCA-protein precipitate.

The precipitate was saved for protein concentration determination while 1.0ml of the supernatant was added to 1.0ml 1% (v/v) phenol and 1.0ml 1.0M Na2CO3, and was incubated at room temperature for 20- 25 minutes. Absorbances were read at 630nm on a Perkin-Elmer (Coleman 124) double beam spectrophotometer and compared to a standard curve made from para-aminophenol (PAP), 0.05mM to 0.001mM.

C. Aminopyrine Demethylase Assay Procedure

0.5ml of aminopyrine stock solution (7.0mg/ml buffer) was added to the reaction mixture instead of 0.5ml of aniline.

The reaction mixture volume remained the same, as did the concentrations of the various reactants. The aminopyrine concentration was 5.10mM.

Reactions were stopped at the appropriate times with 1.0ml 10% (w/v) TCA and centrifuged as above. 1.5ml of the supernatant was combined with 1.5ml Nash's reagent B (15.0g ammonium acetate, 0.3ml acetic acid, 0.2ml acetylacetone, and glass-distilled water to a final volume of 100ml). Incubations were at 60C for 8 minutes in a Dubnoff water bath, 120 oscillations per minute. After cooling to room temperature, absorbances were read at 412nm and compared to a standard curve made from formaldehyde, 0.001% to 0.000125%.

D. Headspace Sampling of the Volatile Aromatic Hydrocarbon Toluene

Reaction mixtures had a total volume of 3.1ml, consisting of 0.1ml aliquotes of toluene stocks in water (50-200 PPM), 2.0ml cofactor (0.372mg NADP, 2.60mg G-6-P, 2.40mg MgCl2 in 2.0ml 0.1M phosphate buffer), and 1.0ml 12,000 x g supernatant equivalent to 100mg liver, wet weight. Reaction mixture concentrations were as follows: 32.3mg liver/ml, 0.162mM NADP, 3.22mM G-6-P, 24.4mM MgCl2, and toluene at 0.0152mM, 0.0228mM, 0.0303mM, 0.0455mM, or 0.0606mM.

Each reaction mixture was incubated at 37C, in the Dubnoff water bath, in a glass scintillation vial (20ml) closed with a customized cap fitted with a 20mm teflon-lined rubber septum. This assured an airtight fit yet allowed sampling

of the gaseous phase with a gas-tight syringe, or a needle attached to a Valco six-port valve. At the end of the incubation period, the sample was cooled to the ambient temperature by immersion in water, and immediately sampled with a Varian 3700 gas chromatograph (hydrogen flame ionization detector) fitted with a six-foot glass column, 0.20mm internal diameter, packed with 10% 1,2,3-tris(2-cyanoethoxy)propane on 100/120 mesh Chromasorb PAW from Supelco, Inc.

After headspace sampling, the protein in each mixture was precipitated with 1.0ml 10% (w/v) TCA, centrifuged, and saved for protein concentration determination as in the other assays.

E. Protein Concentration Determination Using Coumassie Blue G-250

The protein precipitate from each sample of each assay was redissolved in 10.0ml 0.1M KOH, a process usually requiring up to twenty-four hours. After vigorous vortexing to assure a homogeneous solution, an aliquot of this solution was withdrawn and diluted with an equal volume of 0.1M KOH. Then, 0.1ml of this dilution was added to 5.0ml of Coumassie stock (100mg Coumassie Blue G-form, 50ml 95% ethanol, 100ml 85% (w/v) phosphoric acid, and distilled water to a final volume of one liter. After 24 hours, filter with a Whatman #1 filter) and vortexed. Readings were made at 595nm after 5-20 minute incubations at room temperature. Absorbances were compared to a standard curve made from bovine serum albumin (BSA), 10-75ug per 3.1ml.

Results

In early tests, rat livers were homogenized using both the Tekmar, and the teflon and glass Potter-Elvhjem homogenizers. It was determined that no significant differences in enzyme activity were detectable between the two methods. In subsequent experiments, the Tekmar was used exclusively. Trials were also run comparing frozen hepatic microsomal enzyme activity to fresh microsomal enzyme activity: comparing the effectiveness of the cofactor as an NADP-reducing agent when it was prepared daily as opposed to when it had been frozen. In neither case did the freezing of reaction mixture components appear to diminish significantly the enzyme activity, as long as the storage period did not exceed twenty-four hours.

Aminopyrine demethylase activity was readily measurable in all of the rats tested. It was somewhat less pronounced in Pimephales promelas, possibly due to contamination of the samples with bile. Aniline hydroxylase (AH) activity was less measurable in the rat liver microsomes, and was not detectable under present assay conditions in the fathead. Induction of hepatic mixed-function oxidases in rats via the administration of phenobarbital, measurably increased AH activity in the rats. No induction of P. promelas was attempted.

During preliminary headspace sampling of enzymatic activity, it was found to be quite difficult to achieve accepta-

ble reproducibility using gas-tight syringes for injecting the samples into the gas chromatograph. Accuracy improved greatly when a Valco six-port valve was employed in sampling the gaseous phase of the reaction mixture.

To determine if significant binding of toluene with protein in the reaction mixture was occurring without actual metabolism taking place, three methods of running controls were compared. One of these involved samples containing only toluene and water. The second method involved a complete reaction mixture with protein that had been heat denatured at 60C for twenty minutes. The third system replaced the cofactor with phosphate buffer. No significant differences in toluene concentrations were found (Table 6). The third method was ultimately chosen for later experiments in order to standardize the procedure.

TABLE 6

COMPARISON OF TOLUENE-PEAK AREAS RESULTING FROM THE SAMPLING OF THE GASEOUS PHASES OF VARIOUS STANDARD MIXTURES IN ORDER TO DETERMINE IF PROTEIN-BINDING OF THE SUBSTRATE OCCURS. (1)

Standard Mixtures (3)	Peak Area at 3 Concentrations of Toluene (2)		
	A	B	C
I	2170 (26)	1276 (114)	360 (129)
II	2477 (46)	860 (136)	551 (42)
III	2025 (139)	1127 (67)	508 (30)

(1) Peak areas reported as the mean of two samples with the standard deviation in parentheses.
(2) Initial concentrations of Toluene: A=60.6 uM; B=30.3 uM; C=15.2 uM.
(3) Methods of preparing standard mixtures:
I. 0.10 ml Toluene stock (TS) + 3.0 ml water
II. 0.10 ml TS + 2.0 ml cofactor + 1.0 ml heat denatured 12,000 x g supernatant
III. 0.10 ml TS + 2.0 ml KCl/PO4 buffer + 1.0 ml 12,000 x g supernatant

Initial headspace sampling of toluene disappearance was done using a 0.0303mM toluene reaction media. Various incubation times were examined. Disappearance of toluene in the headspace was most rapid and linear between 10 and 20 minutes at 37C, with little or no metabolism noted thereafter.

In a comparison of metabolic activities of rat liver microsomes using different concentrations of toluene (i.e. 0.0606mM, 0.0303mM, and 0.0152mM) the rate of metabolism dropped from 0.0245 nmoles/mg liver (wet weight)/minute at

the highest concentration tested to 0.0163 nmoles/mg liver (wet weight)/minute at 0.0152mM concentrations.

When a rat was induced with phenobarbital at a rate of 100mg/kg body weight per day for three days, the highest rate of metabolism, again at the 0.0606mM concentration, was 0.0328 nmoles/mg liver (wet weight)/ minute. This represented a 33.9% increase in the rate of disappearance of toluene from the reaction medium. The lowest rate of disappearance occurred at the lowest concentration tested (Table 7). Headspace sampling has not yet been attempted in the fathead minnow.

TABLE 7

COMPARISON OF RATES OF LIVER MICROSOMAL ENZYME METABOLISM OF TOLUENE (T) IN CONTROL AND PHENOBARBITAL (PB) INDUCED FEMALE RATS. (1)

Initial T conc (2)	Control	PB-Induced	% Change
A	0.0245	0.0328	34
B	0.0226	0.0290	28
C	0.0164	0.0195	19

(1) Values given in nmoles of toluene metabolized/mg liver (wet weight)/minute of incubation time. n=3

(2) Initial toluene (T) concentrations : A=60.6 uM; B=30.3 uM; C=15.2uM.

VII. Preliminary Results from Work Started Using Scanning Electron Microscopy (SEM) of Control and Toluene Exposed Fathead Minnows.

Studies investigating the effects of toluene upon fathead minnow gross morphology have been initiated. Pilot studies have focused upon gill structure. Adult minnows were exposed in a closed system for 18 hours to 30 PPM toluene. Control minnows were placed in a similar system, except that toluene was not added to the water, for the same length of time. At the end of the 18 hour exposure period, individual minnows were placed in a Petri dish and decapitated. Gills were immediately flooded with cold 5% glutaraldehyde. Following their dissection, they were placed in fresh 5% glutaraldehyde and kept at 4 C for two hours. Following fixation they were carried through three 15 minute rinses in Millonig's phosphate buffer (pH 7.4) and then dehydrated in a graded series of ethanol. This was followed at once by critical point drying with liquid CO2 and a Samdri pvt-3 critical point drying apparatus. Each gill was mounted on a specimen stub with silver paint, and coated with a thin layer of gold in a Hummer 11 sputter coater. Specimens were examined in a JEOL JSM-35 scanning electron microscope. Figure 3 illustrates the typical morphology of the gill as seen in the control group. Perpendicular to the gill arch

(3)

(4)

(A) there projects a double row of gill filaments (F). Only one arch is seen in this figure. Each filament bears two rows of flap-like secondary lamellae (L). These are seen in higher magnification in Figure 4. There is an asymmetry to each filament, in that the respiratory side and the buccopharyngeal side are contoured differently. The surface of both the filament and the lamellae are relatively smooth (Figures 4 and 5). This is in contrast to a previous report (Hinton and Walker, 1980) in which filaments were said to be covered with a layer of microridges, but lamellae had smooth surfaces.

Figure 6 is a higher magnification of secondary lamellae surfaces taken from a minnow which had been treated with toluene. Although the surface remains relatively smooth, there does appear to be some folding of the membrane. Gill filaments and their secondary lamellae after exposure to toluene are also shown in Figure 7. The regularity of the flap-like lamellae, as well as the smoothly contoured filaments, appear to be significantly less in this minnow than in control minnows. Again, this observation is consistant with what has previously been reported for minnows exposed to high concentrations of manganaese sulfate or ferris oxide (Hinton and Walker, 1980).

Additional work will be done in an attempt to confirm these observations and obtain quantifiable data. Attention will be focused on the differences, if any, seen between the

(5)

(6)

(7)

respiratory and the bucco-pharyngeal sides of the gills. SEM studies will be supplimented with TEM and light microscopy.

Literature Cited

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MILLONIG, G. : A Modified Procedure for Lead Staining of Thin Sections. J. Biophys. Biochem. Cytol. 11 736-739. (1961).

VIII. Future Research Plans

A. Finish research on the inter-relationship between solubility of JP-4 alkylbenzenes at four different salinities and five temperatures.

B. Complete and submit for publication data relating to LC 50 and MATC for toluene in fathead minnow embryos, 1-day posthatch larvae, and 30-day old fish.

C. Complete the study on early embryonic development of fathead minnows at 20 and 25 C. This study can be used to determine the effect of toluene on early development.

D. Light microscopic studies of tissue sections of

fathead minnows in control and toluene exposed embryos will continue.

E. Headspace analysis, using GC, will be used to study the metabolism of toluene and other alkylbenzenes by rat and fathead minnow liver microsomes will continue.

F. Perfect a procedure for using HPLC for measuring benzene and toluene metabolism by rat and fathead minnow liver microsomes. C14 labeled benzene and toluene will be used in these studies. The peaks separated on the HPLC column will be collected and counted in a liquid scintillation counter.

G. Scanning electron microscopy will be used for studying gross morphological characteristics in control and toluene exposed fathead minnow larvae and adults.

IX. List of Professional Personnel

J.D. Brammer

R.L. Puyear

C. Medich

E.W. Devlin

F.D. Carter

G. Linz

A. Pathiratne

I. Interaction with other Agencies

Richard Siefert and J.H. McKim, US EPA, Duluth, MN

II. New Discoveries, Inventions, or Patent Disclosures, and Specific Applications Stemming from the Research Effort

None

APPENDIX A

Use of reverse phase C-18 mini-columns for concentrating water-soluble hydrocarbons

R. L. Payear, K. J. Fleckenstein,

W. E. Montz, Jr. and J. D. Brammer

In most cases water-soluble hydrocarbons are present in such low concentrations they cannot be detected without first being concentrated. A variety of methods have been developed to do this, but most are limited in that they require a fairly expensive set-up, are technically difficult and time-consuming to run, or are inefficient. As an example, solvent extraction (ASTM: D2778-70 1979) has often been used to determine trace amounts of hydrocarbons, but in this procedure many of the volatile components are lost during extraction. Consequently, purge and trap methods are frequently used for volatile organics (GROB & ZURCHER 1976). Macroreticular resins such as XAD-2 or XAD-4 and pellicular reverse phase liquid chromatography supports have also been used in the analysis of water-soluble hydrocarbons (JUNK, et al., 1979, CHANG & FRITZ 1978, TATEDA & FRITZ 1978, OGAN, et al., 1978, SANER, et al., 1979), but these methods are either technically difficult or limited as to their general use for different hydrocarbons. Some of these methods for trace enrichment were recently reviewed by DRESSLER (1979).

This study was initiated to find a rapid, simple, yet efficient method of concentrating petroleum-derived water-soluble hydrocarbons. The aliphatics and alkylbenzenes se-

lected for study are those which are the major water-soluble components of the jet fuel, JP-4.

Materials and Methods

The hydrocarbon standards used were analytical grade (Alltech Assoc.). Solvents were spectral grade (Burdick and Jackson). The JP-4 jet fuel was obtained from the North Dakota Air National Guard, Fargo, N.D. Each mini-column (Sep-Pak C-18, Waters & Assoc.) was a polyethylene cartridge filled with a silica pellicular support having octadecylsilane chemically bonded to the surface. These cartridges were designed to fit onto the end of a syringe.

Every Sep-Pak used for concentration of standards or JP-4 from water was attached to a syringe and conditioned with 2 ml ethyl acetate followed by 7 ml glass-distilled water. The sample was applied to the column with a syringe at a rate of about 20 ml/min. Two ml of ethyl acetate was used to elute the sample from the Sep-Pak. These particular volumes were empirically chosen as optimal for quantifiable recovery. Elution of the Sep-Pak with methanol or acetonitrile resulted in a large GC solvent peak which masked many of the compounds of interest. Recovery of the ethyl acetate was enhanced by passing .5 ml of water and 2 ml air through the Sep-Pak. The eluate was then frozen to separate the solvent and aqueous phases. The unfrozen solvent layer containing the ethyl acetate and the water-soluble hydrocarbons was then decanted and the volume measured.

Hydrocarbon-free water was prepared by passing tap water through a conditioned Sep-Pak. The efficiencies of the Sep-Pak in concentrating the selected hydrocarbons were determined by injecting a controlled amount of mixed standards (Table I) into 20 ml of hydrocarbon-free water in a glass syringe. This sample mixture was forced through a conditioned Sep-Pak. Two successive rinses of 20 ml hydrocarbon-free water were each forced through the Sep-Pak. Efficiencies were estimated from a graph of volume of hydrocarbon recovered as a function of volume of each hydrocarbon forced through the Sep-Pak. The slope of the line is reported as the percent efficiency. The same procedure was used to determine Sep-Pak efficiency for the recovery of hydrocarbons in JP-4 (Table 1).

To test the ability of this method to concentrate dilute samples of petroleum-derived water-soluble hydrocarbons from water, 10 ul of JP-4 was mixed slowly in 500 ml of tap water for 2 hours at room temperature. The mixture was then allowed to stand for 2 hours in a separatory funnel. 450 ml of the aqueous phase was then drawn off and put through a conditioned Sep-Pak with a glass syringe. The hydrocarbons retained on the Sep-Pak were eluted as described above.

Hydrocarbons were detected using a Beckman GC 45 gas chromatograph with a flame ionization detector. A stainless steel chromatographic column (2 mm i.d. by 2.4 m) was packed with 10% 1,2,3-tris (2-cyanoethoxy) propane (TCEP) on 100/200 mesh Chromosorb PAW (Supelco, Inc.).

Table 1. Percent recovery of single and mixed hydrocarbon standards and JP-4 from water using Sep-Pak C_{18} cartridges.

	Single Hydrocarbon Standard a)	Single* Hydrocarbon Standard a)	Standard* Hydrocarbon Mixture a)	JP-4* b)	JP-4 c)
Aliphatics				90	94
hexane			75		
heptane			74		
octane			69		
decane			61		
Alkylbenzenes					
toluene	80	88	73	71	60
n-propylbenzene			87	77	61
ethylbenzene	50	75	82	66	35
m- & p-xylene	42	74	87	69	40
isopropylbenzene			85		10
o-xylene			68	73	35
3- & 4-ethyltoluene			86	70	33
2-ethyltoluene			84	73	32
1,2,4-trimethylbenzene			86	66	33
1,2,3-trimethylbenzene			71	65	37
Average recovery of hydrocarbons			78	72	43

* in ethyl acetate

Concentration of hydrocarbon placed in a glass syringe containing 20 ml of water and concentrated with a Sep-Pak ranged from: a) 1-150 PPM, b) 10-500 PPM, and c) 50-1500 PPM. These concentration ranges were selected so that they nearly equalled that found in JP-4 in b) or in c) so that quantitative amounts of JP-4 could be delivered.

The water-soluble hydrocarbons from JP-4 were identified by comparison with retention times of standard mixtures and by peak enhancement methods. Concentrations were calculated from peak areas using the external standard method with a Spectra Physics SP-4000 data system. A mixture of standards (Table 1) was used for calibration and to determine the response factors for each hydrocarbon used. Individual response factors were used to convert respective peak areas to concentrations in PPM by volume (ul/l). For each hydrocarbon tested a range of concentrations was used to obtain a line so that the slope could be calculated over a range of 1-500 PPM, which gave linear recovery. This slope multiplied by 100 yielded percent recovery.

Results

The reproducibility between individual Sep-Paks of the same lot number averaged +5%. All values tabulated are the average results of duplicate runs. Reproducibility of injections and quantitation was +2.6%. The efficiency of recovery depended upon the total amount of hydrocarbon forced onto the Sep-Pak and not upon concentration. The two different lots of Sep-Paks used in this study gave average recovery efficiencies of hydrocarbon standards of 78% and 63%.

Tests were performed to determine the fate of the hydrocarbons not recovered from the Sep-Pak. This was done by placing six Sep-Paks in a series with a one inch glass tubing connector between each pair of Sep-Paks. A mixture of

JP-4 in water was then forced through the series and each one was eluted as described previously, with ethyl acetate. All of the alkylbenzenes recovered were in the first two Sep-Paks. Aliphatics, however, were recovered in small amounts even in the last Sep-Pak in the series. When the same Sep-Paks were then eluted with hexane, the only component which came off any of them was toluene.

Data obtained regarding the capability of a Sep-Pak to concentrate hydrocarbons from water is presented in Table I. When hydrocarbons were analyzed singly, Sep-Pak recovery efficiency was greater than when more complex standard hydrocarbon or JP-4 mixtures were analyzed. The use of ethyl acetate as a solvent for the addition of the single standards, standard hydrocarbon mixture or JP-4 improved the ability of the Sep-Pak to concentrate hydrocarbons from water.

Concentrations of JP-4 in ethyl acetate were chosen so that no more than 300 ul of ethyl acetate was put into the water to be forced onto the Sep-Pak. With larger volumes of ethyl acetate lower recovery efficiencies were noted. This may be because the greater amount of ethyl acetate forms a layer on top of the water. When this ethyl acetate is forced through the Sep-Pak after the water, it may elute the hydrocarbons retained on the Sep-Pak. The concentration range for JP-4 in water was chosen so that the concentrations of the individual major components of JP-4 were compa-

rable to the concentration range for the single hydrocarbons (1-1500 PPM). These concentrations were all based on amount of hydrocarbon of interest per 20 ml of water.

An example of the capability of the Sep-Pak to concentrate a dilute sample of hydrocarbon from water is demonstrated by the chromatogram in Figure 1. Under the chromatographic conditions used the aliphatics were not well resolved. The alkylbenzenes, however, were well separated and quantitation of each component was possible.

Discussion

Sep-Pak C-18 cartridges from a single lot gave consistent recovery efficiencies for the hydrocarbons investigated over a range of 1-1500 PPM in 20 ml of water (Table 1). Different lots of Sep-Paks, however, gave significantly different recovery efficiencies for the same compound. SANER, et al. (1979) found the same variation using toluene and benzene in water. For samples containing more than 1500 PPM, we found that volumes less than 20 ml should be processed. On the other hand, larger volumes of more dilute samples would give a measurable recovery. SANER, et al. (1979) describe a method for calculating the optimum sampling volume for benzene in water. They observed that when large volumes of a dilute solution were passed through a Sep-Pak the trapping efficiency was reduced, perhaps as a result of the high flow rate they used for loading the Sep-Pak.

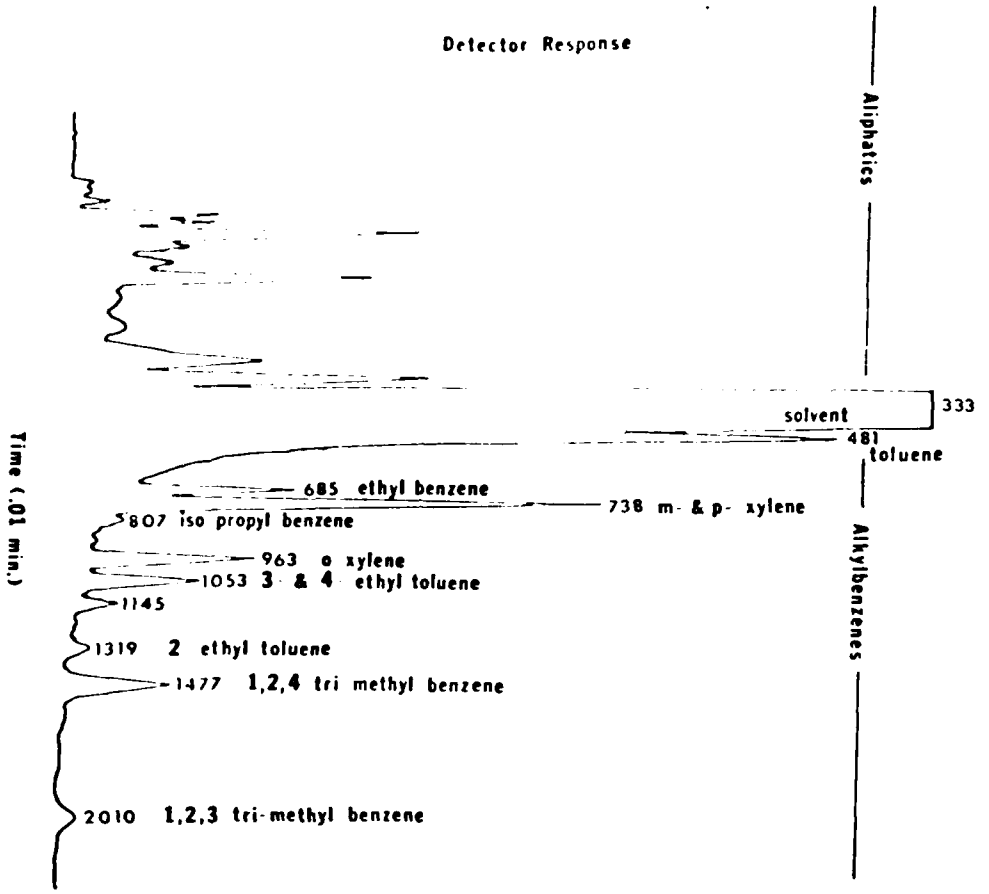

Fig 1. GLC of the water-soluble fraction of JP-4 in water. 10 µl JP-4 in 500 ml of water was mixed for 16 hours and concentrated with a Sep-Pak C18. RT (.01 min), detector: FID, 5 x 10^{-12} A full-scale.

Data in Table 1 indicates single hydrocarbons are generally trapped more efficiently than when present in a mixture of hydrocarbons. This has been seen by numerous other investigators using various types of macroreticular (XAD) resins and several column designs (JUNK, et al., 1974, STEPHEN & SMITH 1977, CHANG & FRITZ 1978, TATEDA & FRITZ 1978, VAN ROSSUM & WEBB 1978, DRESSLER 1979, SAMER et al., 1979, and JANARDAN & SCHAEFFER 1980). Care must be used when applying trapping efficiencies obtained from analysis of single hydrocarbons to experimental samples in which a mixture of hydrocarbons is present. If the trapping efficiency used is unrealistically high, the estimation of the concentration of the hydrocarbon under study will be lower than what is actually present in the sample.

Data in Table 1 also indicates that the efficiency of trapping of the solutes by the Sep-Pak is improved by the addition of a small amount of organic solvent. Several possible explanations can be advanced to explain this result, including: (a) the organic solvent increases the solubility of the solute in water, (b) it alters the hydrophobic interface between the packing material in the Sep-Pak sufficiently so the material can interact with the solute in water, (c) the solvent reduces the loss of solute on the barrel and plunger of the syringe used to push the sample through the Sep-Pak, or any combination of the above options.

SAMER, et al., (1979) report that the order in which benzene and toluene are concentrated from a water sample with a Sep-Pak influences the trapping efficiency of the Sep-Pak. In a water sample containing a number of components the order in which any component is added cannot be controlled and therefore all components must be concentrated at one time. In the present study, hydrocarbon samples were added to water either individually, in a mixture of 14 compounds, or as JP-4 jet fuel (Table 1). There was variability in the ability of the Sep-Pak to concentrate aliphatics from water. This could have been due to their rather low solubility in water and higher volatility. Recovery of the aliphatics in JP-4 was greater than in the standard mixture.

Efficiency of the Sep-Pak for concentrating alkylbenzenes from water varies. Toluene is more efficiently trapped when it is the only component present. Ethylbenzene and m- & p-xylene are not as efficiently trapped when they are present independently. When the hydrocarbon matrix is complex, as with JP-4, the trapping efficiency is less than in the less complex mixture. The overall average efficiency of recovery of the standard hydrocarbon mixture is somewhat greater than for JP-4. There was a great difference in recovery between the JP-4 added to water in ethyl acetate and JP-4 added directly to water. These observations indicate care should be exercised when drawing conclusions about the efficiency of XAD, Sep-Pak or other trapping matrices and that trapping

efficiencies for single organic compounds are not equivalent to those found when multi-component mixtures are involved.

The chromatogram in Figure 1 shows that a Sep-Pak can be used to concentrate a relatively dilute sample of JP-4 mixed with water. When the Sep-Pak is to be used to concentrate hydrocarbons from more dilute solutions of JP-4 in water larger volumes of water must be processed. When larger volumes of water are forced through a Sep-Pak, evaluation and quantitation of data should be done with caution. SANER, et al., (1979) report that benzene and toluene are lost from the Sep-Pak when large volumes (more than 150 ml) of seawater are passed through the Sep-Pak.

In this work, a Sep-Pak has proven to be a useful tool for concentrating aliphatics and alkylbenzenes from water. It was 5 to 10% less efficient than XAD, as reported by DRESSLER (1979), for concentrating some of the same aromatics. This however is not a great reduction in efficiency when the ease and convenience of using these cartridges is considered. The C-18 packing material used in the Sep-Pak is rather hydrophobic. A further extension of the usefulness of this technique would be to use a mini-column packed with more polar bonded phase such as C8 or C2. These columns could be used to concentrate the more water soluble organics from water.

Acknowledgments

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APPENDIX B

THE IDENTIFICATION AND QUANTIFICATION OF WATER-SOLUBLE HYDROCARBONS GENERATED BY THE OPERATION OF TWO-CYCLE OUTBOARD MOTORS

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Abstract. A 7.0 horsepower (HP) and a 10.0 HP outboard motor were operated at 3,500 ± 200 revolutions-per-minute (rpm) and 1,700 ± 200 rpm respectively for 30 min in a 160 L tank of tapwater. Exhaust hydrocarbons were concentrated by passage through a C_{18} reverse phase extraction column, and then eluted with either ethylacetate or acetonitrile. Gas-liquid (GLC) and/or high performance liquid (HPLC) chromatography analyses were used for identification and quantification of nine hydrocarbons. Identities were confirmed for sever of these using GLC/mass spectrometry. Four additional hydrocarbons were tentatively identified with these procedures. Aromatic compounds composed the majority of the hydrocarbons detected; only a few aliphatics were

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present and those in trace amounts.

In the U.S. during 1977, 900,000 new outboard motors were purchased, with a total of approximately 7 million motors in use. Average size of the motors was 44.4 horsepower (HP) (U.S. Census Bureau 1979). Compared with other types of internal combustion engines, outboard motors are inefficient, releasing significant amounts of raw fuel mixture and of fumes into the water (Muratori 1968; Stewart and Howard 1968; Ferrer 1970; Jackiviez and Kuzminski 1973).

The chemical composition of gasolines used in outboard motors, as well as of other refined fuels and crude oils, has been extensively studied (Howard and Ferguson 1961; Martin and Winters 1963; Schwartz and Brasseaux 1963; Snyder et al. 1963; Sanders and Maynard 1968; Coleman et al. 1973; Papazova and Pankova 1975; DiCorcia et al. 1978). Because there is a substantial amount of gasoline in the exhausts of outboard motors, these exhausts contain many of the same hydrocarbons that are found in gasolines and oils. English et al. (1963) identified major pollutants in exhausts as nonvolatile oils (the lubricating oils in the fuel mixture), volatile oils (the gasoline in the fuel mixture), phenols, and lead. They did not identify specific hydrocarbons, nor classes of hydrocarbons present. In an unpublished progress report (Kuzminski et al. 1973) six hydrocarbons were identified as water-soluble components of

outboard motor exhausts, but quantification of these compounds was not accomplished. Eschwend (1979) examined the temporal variations of volatile organic compound concentrations in coastal seawater from Vineyard Sound, Mass. He found the C_2- and C_3-benzenes were the most persistently abundant compounds and that their concentrations were 2 to 10 times higher than average immediately after summer weekends in which tourist and recreational activities were greatest. This corresponded to periods in which motorboats were most heavily used. Studies to determine the compounds in outboard motor exhausts are needed (Jackivicz and Kuzminski 1973; Liddle and Scorgie 1980). Because information on outboard motor exhaust composition was lacking, this study focused upon identification and quantification of water-soluble hydrocarbons in these exhausts.

Materials and Methods

Outboard Motors

Two two-cycle outboard motors were selected for study. One was a 7.0 HP 1972 single cylinder, air-cooled motor with a recycling system for re-ignition of unburned crankcase material. The other was a 10.0 HP 1954 two cylinder, water-cooled motor which exhausted crankcase residuals directly into the water. Both motors used the same 50:1 gasoline:oil

fuel mixture and were in good operating condition. The gasoline used was regular grade fuel obtained locally; a 50:1 two-cycle motor lubricant was used.

Tests with the two motors were run separately. Each motor was placed into a clean stainless steel tank filled to 160 L with tapwater and run for 30 min. The 7.0 HP motor was operated at 3,500 ± 200 rpm for 30 min. The 10 HP model was run at 1,700 ± 200 rpm because operation at higher rpm's resulted in excessive agitation and water loss from the tank. Fuel consumption was measured volumetrically.

Sample Preparation

Water samples were taken from the bottom of the tank via a spigot and filtration through a 3 nm Millepore filter followed by a 0.22 nm Millepore filter was started immediately to remove emulsified components. Hydrocarbons remaining in the aqueous phase after filtration were designated water-soluble fractions. All samples were kept refrigerated until analysis, which was completed within 8 h after collection.

Water-soluble fractions were concentrated using a column containing pellicular silica coated with octadecasilane (C_{18}, available from Waters Associates, Milford, MA under the tradename Sep-Pak). Before use each column was conditioned to the aqueous phase before use by

passing 1 ml of ethylacetate, followed by 5 ml of distilled water through the column. Exhaust water samples (400 to 600 ml) were then forced through the column. Hydrocarbons were then eluted with 2 ml of ethylacetate or acetonitrile. Since solvent peaks frequently mask unknown peaks, the use of two solvents, each having a different retention time, was necessary. All solvents used were spectral grade (Burdick and Jackson, Muskegon, MI). During filtration, a Sep-Pak was placed in the vaccuum line to collect volatiles which might have been lost from the sample. These were eluted in the same manner as the dissolved hydrocarbons.

Hydrocarbon Concentration Determinations

A Beckman Model GC 45 GLC equipped with a flame ionization detector was used for some analyses. A 244 cm stainless steel column (2 mm I.D.) packed with 10% 1,2,3-TRIS(2-cyanoethyoxy)propane on 100/120 Chromosorb P.A.W. (Supelco, Inc., Bellefonte, PA) was used. Pertinent operating conditions were N_2 column = 20 cm^3/min, H_2 = 44 cm^3/min, air = 240 cm^3/min, carrier makeup = 60 cm^3/min, inlet temperature = 170°, detector temperature = 170°, and detector line temperature = 205°. All analyses were performed isothermally at 80° $\pm$ 2°. Sample injection volumes were 0.2 ul.

An Altex Model 322 MP HPLC was also used. The column (25 cm long with 4.6 mm I.D.) was packed with Lichrosorb C_{18} stationary phase on 10 micron support material (Altex Associates, Berkeley, CA). The analytical column was protected by a guard column packed with Chromosorb LC-4 coated with a C_{18} stationary phase (Johns-Manville, Denver, CO). A 70:30 ratio of acetonitrile and water at a flow rate of 1.5 ml/min was used to elute the column. Peaks were detected with a fixed wavelength ultraviolet detector operated at 254 nm. Samples were injected onto the chromatographic column with a 20 ul fixed loop injection valve.

Data from both chromatographs were fed into a Spectra-Physics SP4000 data system and quantified using an external standard program. Calibration was checked daily and tapwater and solvent blanks were run and subtracted from sample concentrations. Identification of the components was was determined, in part, by comparison of retention times of the unknowns with those of known standards (Alltech Associates, Arlington Heights, IL) and by peak enhancement techniques.

To obtain molecular weights of the unknown compounds, 0.5 ul aliquots of the sample eluate were injected into a Varian/MAT 112S double focussing magnetic mass spectrometer equipped with a Varian 3700 gas chromatograph. A glass

column 274 cm long by 2 mm I.D. was packed with 1,2,3-TRIS(2-cyanoethoxy)propane on 100/120 P.A.W. silica. The mass spectrometer and gas-liquid chromatograph were interfaced with a glass jet-faced separator. The data system employed was an SS 200 Varian Spectral System. Pertinent operating data were: He carrier = 24 cm^3/min, injector temperature = 150°, column temperature = 80°, detector temperature = 200°, and separator temperature = 190°.

Results

Figure 1 shows a gas-liquid chromatograph of the hydrocarbons present in exhausts from the 7.0 HP motor. Chromatographs similar in pattern, but with larger peak areas, were obtained from 10.0 HP motor exhausts. Nine different peaks, corresponding to nine individual water-soluble hydrocarbons were detected using GLC. Three additional peaks, each corresponding to a pair of hydrocarbons, were also detected using GLC. The concentration of benzene (peak not shown in Figure 1) was determined by separate chromatographic analyses of concentrated exhaust hydrocarbons eluted with acetonitrile. Identified compounds and their concentrations are listed in Table 1. Individual detector response factors were employed in determining concentrations for each compound.

Table 1. Water-soluble hydrocarbon concentrations produced by operating 7. outboard motors for 30 min in 160 L of tapwater

Compound	Concentration (ppm, vol:vol) 7.0 FP[a]	10.0 HP[b]
trace n-decane, n-dodecane, and unknowns	7.78 ±0.37	9.39 ±0.28
methylbenzene	1.19 ±0.12	2.04 ±0.06
n-propylbenzene	0.03 ±0.00	---
ethylbenzene	0.09 ±0.10	2.19 ±0.03
1,3-diethylbenzene and 1,4-diethylbenzene	2.27 ±0.19	7.66 ±0.13
isopropylbenzene	0.10 ±0.00	0.65 ±0.02
1,2-dimethylbenzene	1.49 ±0.07	5.70 ±0.12
1-methyl-3-ethylbenzene and 1-methyl-4-ethylbenzene	2.48 ±0.18	9.25 ±0.17
1-methyl-2-ethylbenzene	1.04 ±0.04	3.70 ±0.10
1,2,4-trimethylbenzene	7.88 ±0.56	24.24 ±0.89
1,2,3-trimethylbenzene	1.93 ±0.03	5.04 ±0.12
benzene	0.23 ±0.03	7.58 ±0.58
unknowns	5.63	21.64

[a]Values expressed as the mean ±1 standard deviation of 7 GLC analyses
[b]Values expressed as the mean ±1 standard deviation of 5 GLC analyses

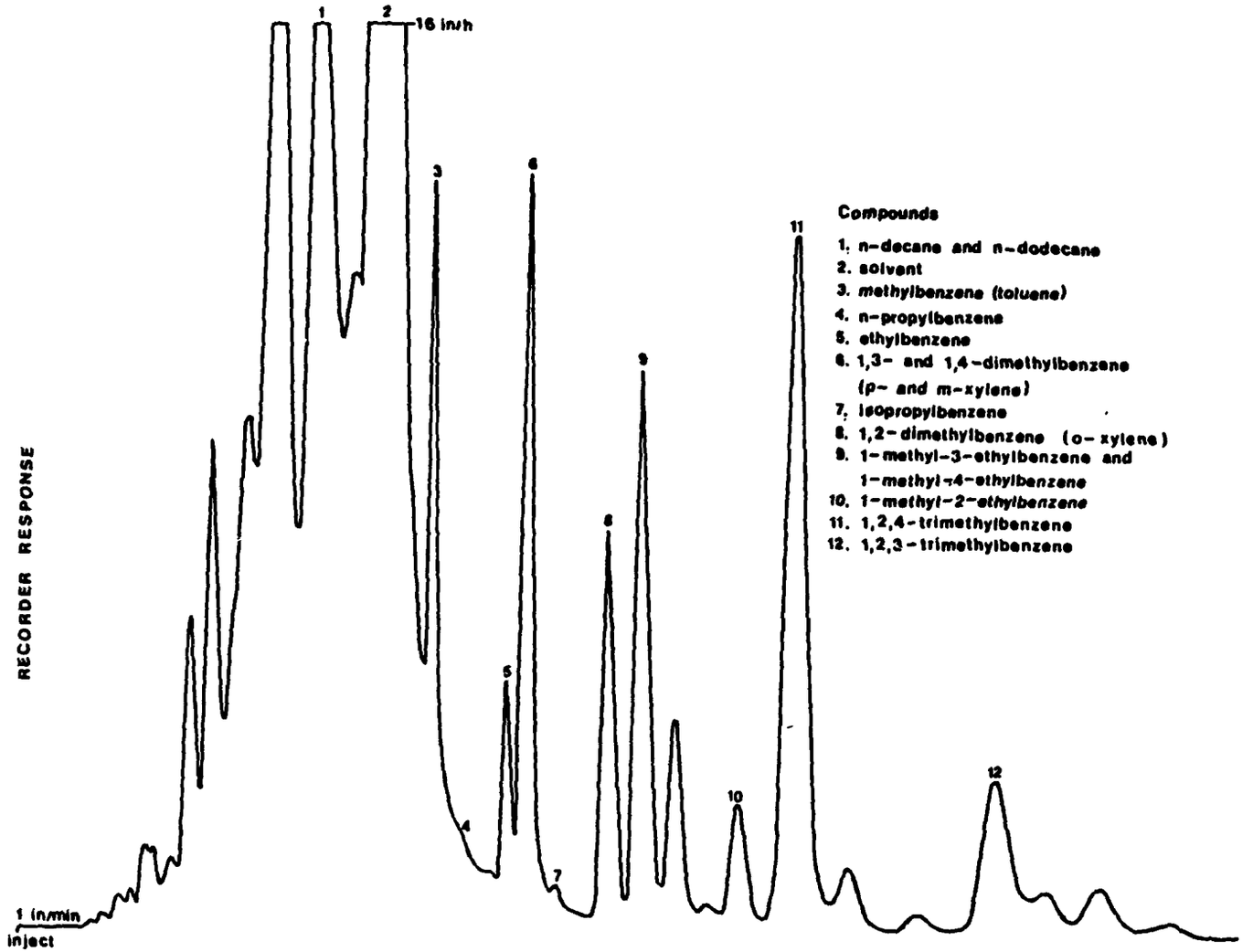

Fig. 1. GLC of exhaust hydrocarbons generated by a 7.0 HP outboard motor run for 30 min at 3500 rpm. Flame ionization detector sensitivity = 1 x 10^{-12} amps full scale output. Chart speed = 1 in/min at injection and changed to 16 in/h after the solvent was eluted.

Table 1. Water-soluble hydrocarbon concentrations produced by operating 7.0 and 10.0 HP outboard motors for 30 min in 160 L of tapwater

Compound	Concentration (ppm, vol:vol) 7.0 HP[a]	10.0 HP[b]	Method of detection
trace n-decane, n-dodecane, and unknowns	7.78 ±0.37	9.39 ±0.28	GLC
methylbenzene	1.19 ±0.12	2.04 ±0.06	GLC,HPLC,MS
n-propylbenzene	0.03 ±0.00	---	GLC,HPLC
ethylbenzene	0.09 ±0.10	2.19 ±0.03	GLC,MS
1,3-diethylbenzene and 1,4-diethylbenzene	2.27 ±0.19	7.66 ±0.13	GLC,HPLC,MS
isopropylbenzene	0.10 ±0.00	0.65 ±0.02	GLC
1,2-dimethylbenzene	1.49 ±0.07	5.70 ±0.12	GLC,HPLC,MS
1-methyl-3-ethylbenzene and 1-methyl-4-ethylbenzene	2.48 ±0.18	9.25 ±0.17	GLC,HPLC,MS
1-methyl-2-ethylbenzene	1.04 ±0.04	3.70 ±0.10	GLC,HPLC,MS
1,2,4-trimethylbenzene	7.88 ±0.56	24.24 ±0.89	GLC,HPLC,MS
1,2,3-trimethylbenzene	1.93 ±0.03	5.04 ±0.12	GLC,HPLC,MS
benzene	0.23 ±0.03	7.58 ±0.58	GLC,HPLC,MS
unknowns	5.63	21.64	

[a]Values expressed as the mean ±1 standard deviation of 7 GLC analyses
[b]Values expressed as the mean ±1 standard deviation of 5 GLC analyses

Hydrocarbons not identified were assigned intermediate response factors for purposes of quantification. Values reported in Table 1 take into account the different recovery efficiencies previously determined for each compound (Puyear et al. 1981). Unknowns comprized 17% and 22% of the total exhaust hydrocarbons from the 7.0 HP and 10.0 HP motors, respectively. Similar concentrations for the aromatic compounds were obtained using HPLC.

Mass spectral analysis verified the identity of methylbenzene (toluene), n-propylbenzene, 1,2-dimethylbenzene (o-xylene), 1-methyl-2-ethylbenzene, 1,2,4-trimethylbenzene, 1,2,3-trimethylbenzene and benzene. Tentative identification was made for four other hydrocarbons. 1,3-dimethylbenzene (p-xylene) and 1,4-dimethylbenzene (o-xylene) which had identical molecular weights and, using our chromatographic procedures with 1,3- and 1,4-dimethylbenzene standards, identical retention times. Thus we could not determine whether one or both of these compounds were present in peak number 6 Figure 1. We were able to determine the total concentration which is reported in Table 1. Peak number 9 (Figure 1) also contained either one or both of a pair of hydrocarbons (1-methyl-3-ethylbenzene, 1-methyl-4-ethylbenzene) having the same molecular weight and identical retention times. Again, the presence of one or both of these compounds was

suggested by mass spectrometry. A third peak, number 1 in Figure 1, was thought to contain n-decane and n-dodecane because the retention time of this peak matched that of corresponding standards. The identity of the compounds in peak number 1 could not be verified by mass spectrometry. This peak also included substantial quantities of unidentified hydrocarbons since the values obtained exceeded the combined solubility of both compounds in water.

Discussion

Sep-Paks provide substantial improvement over sample concentration procedures used in previous studies. The columns allowed samples to be concentrated in minutes rather than hours and did not require the application of heat or evaporation with inert gases. Because of this, fewer volatiles were lost than with earlier procedures. Our data indicated that, at least for the hydrocarbons tested, all were recovered with efficiencies ranging from 46 to 69% (mean = 62%) (Montz 1980; Puyear et al. 1981). Because recoveries of these compounds were relatively equal, Sep-Paks are versatile in concentrating and recovering complex mixtures of unknown hydrocarbons from aqueous samples.

The design of two-cycle outboard motors allows a wide variety of petroleum hydrocarbons to be exhausted into water because intake and exhaust strokes are combined (Muratori

1968). This results in inefficient operation (Stewart and Howard 1968). In these motors, the intake and exhaust valves are open during overlapping periods of time so that the fresh fuel-air mixture can pass out the exhaust port without being combusted. Thus, many of the water-soluble hydrocarbons from gasolines are also found in outboard motor exhaust.

Nine distinct compounds in outboard motor exhaust were identified as water-soluble hydrocarbons. Four others were tentatively identified. Of these, the aromatics were present in highest concentrations. In comparison, refined gasoline composition is primarily aliphatic in nature. Aliphatics were probably not present in substantial quantities in exhaust water samples because of their low solubility in water (Shaw 1977). Our qualitative analyses have shown that the monocyclic aromatics are present in highest concentrations, and these compounds are toxic to biological systems (Penville and Korn 1977).

Although not fully definitive, mass spectral fragmentation patterns appeared which were characteristic of oxygen containing compounds. Those hydrocarbons not identified in Figure 1 were probably partial oxidation products from the combustion of outboard motor fuels. Partial oxidation results in the release of phenols, alcohols, aldehydes, esters, ketones, and acid derivatives

(Kuzmirski et al. 1973). The chromatographic data indicated these compounds were present in relatively small concentrations.

Studies on the biological effects of outboard motor exhausts have shown a variety of deleterious effects can occur (Jackiviez and Kuzmirski 1973; Clark et al. 1974). These studies have not clearly identified specific toxic compunds. We report here specific hydrocarbons and their concentrations of some of the water-soluble components of outboard motor exhausts. Additional studies into the biological role of these compounds are underway.

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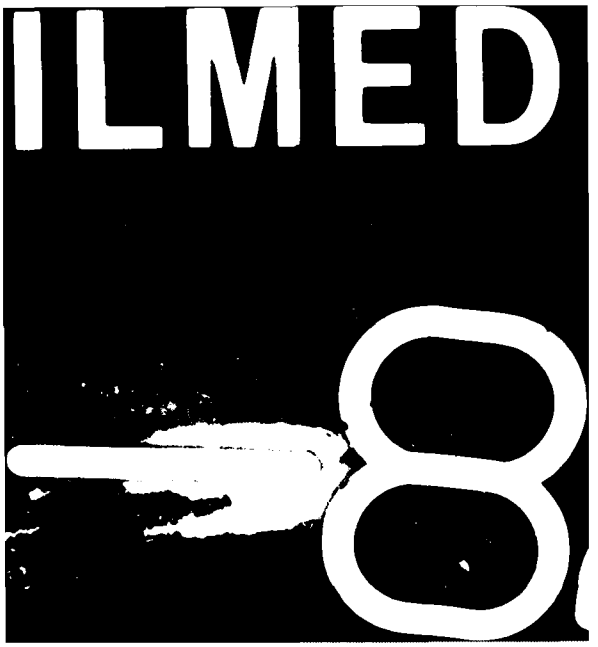
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