COMPARISON OF BIOMONITORING TECHNIQUES FOR EVALUATING EFFECTS OF JET FUEL ON BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*)

by

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DEDICATION

This dissertation is dedicated to my father in memory of all the memories that he left me. I will always remember all of the love, encouragement, and support and the way he and my mother showed their pleasure in my accomplishments. For all of that love and support that was always so evidently there..... Thank you Dad!

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I

INTRODUCTION

Jet fuels such as JP-4 are used by both commercial and military aircraft. There is always a potential for environmental exposure to fuels from leakage during transport, fueling operations and mid air dumping by aircraft making emergency landings. Once this fuel is inadvertently released into the environment it can either directly or indirectly affect aquatic ecosystems. The potential lethal and sublethal effects of jet fuel can then become of great environmental concern.

There have been several studies on the lethal and sublethal effects of jet fuel and its water soluble fraction (WSF) on different species of fish (Cooper et. al. 1981; Jenkins et. al. 1977; Latendresse and Fisher, 1983). Unfortunately, due to problems inherent in working with highly volatile compounds such as WSFs, there have been very few dynamic (constant concentration) studies, thereby limiting their practical application.

The use of fish to serve as biomonitors to determine the effects of pollutants on the aquatic environment is an accepted aquatic toxicology methodology. A recent review (Cairns and van der Schalie, 1980) has shown relatively few

biomonitoring techniques in practical use today; perhaps due to the cost and lack of legally mandated requirements. They also pointed out that direct measurement of sublethal responses is a valid methodology for extrapolating information on the No Observed Effects Level (NOEL) for hazard evaluation. Collection of quantitative sublethal information is increasingly necessary to determine true effects of pollutants on organisms and ecological systems. Collection of such data with computer assisted equipment generates information bases that are statistically sound (Cairns, 1981)

A computer assisted biomonitoring technique successfully used to illustrate sublethal effects of pollutants using respiratory or ventilatory movements was developed by Cairns et al. (1970). Measurement of respiratory movements was shown to be a valid measurement of actual ventilatory rates (Heath, 1972). Ventilatory measurement techniques were used by Thomas and Rice (1979) to demonstrate effects of crude oil on ventilatory rates in salmon.

Another biomonitoring procedure used to investigate effects of sublethal levels of suspected toxicants is preference/avoidance behavior (Cherry and Cairns, 1982). The determination of whether or not fish will actively avoid pollutants is an important consideration in evaluating their

potential environmental impact, i.e. death or organisms, loss of habitat, etc. Such studies have been conducted by Weber et al. (1981) using petroleum hydrocarbons in in-situ experimentation. These studies found differences in behavior that were related to age of salmon. Juvenile salmon being more sensitive to petroleum hydrocarbon contamination when migrating downstream than were adult salmon which were migrating upstream to spawn.

Physiological measurements are useful in establishing affects of toxicants on organisms. Human blood parameters have been studied for many years and evaluation of poikilotherm blood was recommended over three decades ago (Smith et al. 1952). There have been many problems with blood studies in that insufficient background data are available for "normal" levels of blood constituents for most species of fish; consequently, it is impossible to determine deviations or their meaning (Warner et al., 1979). The concept of using specific enzymes as indicators of petroleum pollution have been used by Payne and Penrose (1978) and Rutherford et al. (1979) with mixed results.

No comparative, simultaneous studies have used a series of these state of the art sublethal evaluative techniques on one toxicant to compare results and determine which might be most useful and descriptive of problems occuring in affected

organisms. This research, which is the first part of a study of petroleum and shale derived JP-4 jet fuels, attempts to fill this gap.

II

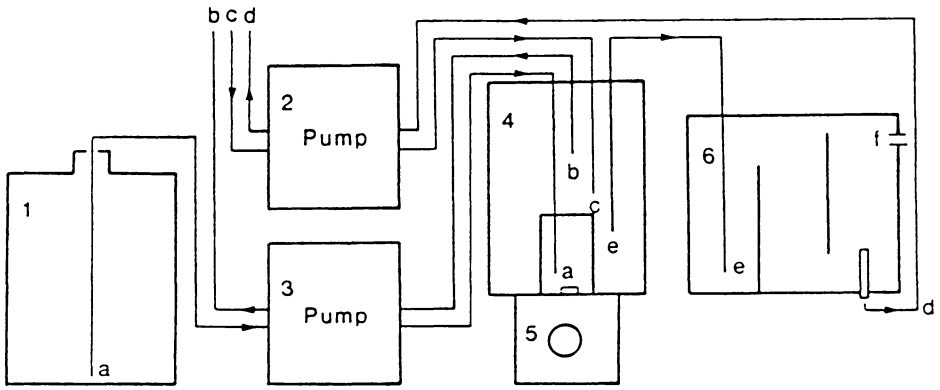
MATERIALS AND METHODS

2.1 GENERATION AND ANALYSIS OF WATER SOLUBLE FRACTION (WSF)

JP-4 fuel for this research was obtained from the Fuels Laboratory, Wright-Patterson AFB, Ohio. A flow-through system similar to that of Dauble et al. (1981) was constructed to generate WSF JP-4. It consisted of a mixing chamber, settling chamber and accompanying pumps for jet fuel and water (Figure 1). The mixing chamber consisted of a glass battery jar with a smaller beaker attached with silicon in the center; it was set on a magnetic stirrer with the stirring bar in the inner beaker. Jet fuel was pumped in at approximately a 5% vol:vol ratio with carbon dechlorinated tap water. Floating ("spent") JP-4 was removed from the mixing chamber at a rate equivalent to fresh JP-4 delivery. Air from an aquarium pump was injected into the beaker to aid in mixing and oxygenation of the mixture. WSF JP-4 flowed from the mixing chamber to the settling chamber, a rectangular glass box with baffles, where any entrained jet fuel was separated. The mixing and

settling times were approximately 1 h depending on flow rates of fuel and water. The fractionator was capable of delivering 150 ml min^{-1} WSF JP-4. The WSF JP-4 was either pumped or allowed to flow by gravity to the experimental equipment. This fractionator was used for all experiments except for the preference/avoidance studies where higher flow rates required a larger volume batch system. The batch system used two-55 gal drums in which a 5% JP-4 solution was mixed for 6 to 12 h. After allowing the mixture to settle for at least 6 h, the initial WSF concentration was determined and the solution was used in the experiments.

Toluene and benzene, the largest and most volatile components of WSF JP-4, were used as markers to calculate WSF concentration (Figure 2). WSF's are defined as a per cent of maximal WSF (MaxWSF) and were based on relative toluene and benzene peak heights. The assumption was made that since these markers were the most volatile fractions of the WSF JP-4 that if they were present in significant concentrations then there was no significant loss of other components. These more volatile components are also the most toxic (e.g. Morrow, 1975). A standard MaxWSF for this study was defined as that concentration resulting from very constant speed mixing of a 5% JP-4 and carbon dechlorinated tap water sample for 3 hours at 25°C. This mixture settled for three hours before extraction.



WATER SOLUBLE FRACTIONATOR

Legend: Jet fuel flows from tank 1 through line a via pump 3 to mixing chamber 4. Water flows through line c via pump 2 to chamber 4. WSF flows from chamber 4 to settling chamber 6 through e and from there through d to research equipment via pump 2. Excess fuel is removed from 4 through b via pump 3. Excess WSF flows out of f to waste.

Figure 1: Water soluble fractionator used for exposing bluegill.

To analyze for benzene and toluene markers, procedures modified from those of Fisher et al. (1984) were followed. A 100 ml water/WSF sample was collected in a volumetric flask and 1 ml of hexadecane was added. The flask was vigorously shaken for 3 min and allowed to settle for another 3 min. A 6 ul subsample of the separated hexadecane layer was analysed by gas chromatography.

A Varian 1600 gas chromatograph with a 3 m SE30 column and flame ionization detection was used to analyze WSF JP-4. The chromatograph was programmed to maintain 40^{0}C for 5 min and then increase by 10^{0}C min^{-1} until a final temperature of 200^{0}C was reached. The injector was set at 220^{0}C and the detector at 225^{0}C. Helium, the carrier gas, was delivered at 20 ml min^{-1}; hydrogen and air for the flame were delivered at 30 ml^{-1} and 300 ml^{-1}, respectively.

Standards were made for toluene and benzene as well as several other known constituents of WSF JP-4, e.g. three different xylene isomers and napthalene, using pure chemicals obtained from Fisher Scientific. Standards were used to determine their detention time in the GC column and relative concentrations in WSF JP-4.

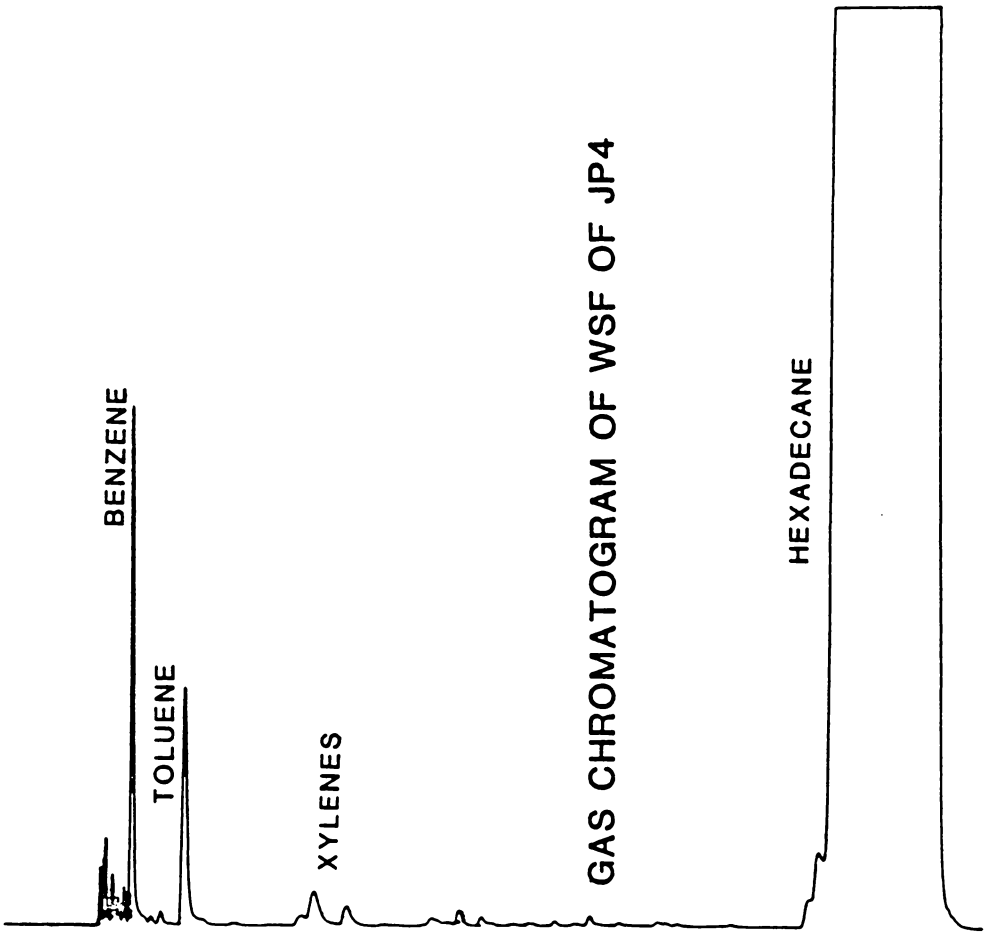


Figure 2: Typical chromatograph for saturated solution of WSF JP-4.

2.2 EXPERIMENTAL ORGANISMS

Juvenile bluegill sunfish obtained from the Kurtz Fish Hatchery in Elverson PA were 4.90 cm (SD±0.20) in standard length (SL) and 3.53 g (SD±0.44) in weight. These fish were used for the toxicity and preference/avoidance testing.

Adult bluegill, required to provide sufficient blood for analysis, were seined from a small, closed pond on the grounds of the Veterans Administration Hospital in Salem VA. The average SL of these fish was 11.18 cm (SD±0.48) and the average weight was 42.93 gm (SD±5.77). Smaller adults (c. 5.0 cm SL) were also seined from the hospital pond for use in ventilatory studies. All fish were placed in laboratory holding tanks, prophylactically treated with tetracycline, and held for at least two weeks before use.

Lethality tests verified the hypothesis that there were no significant differences in the acute responses of the two fish populations and three age groups. (See Section 3.5.)

Carbon dechlorinated tap water was used throughout this research for holding the fish and as a diluent for WSF JP-4. The average pH was 8.17 (SD±0.01; range from 7.6 to 8.7), average hardness was 58.72 ppm (as $CaCO_3$, SD±10.88), average alkalinity was 36.02 ppm (as $CaCO_3$, SD±8.70), and average conductivity was 1.193 mv (SD±0.11).

2.3 TOXICITY OF JP-4 JET FUEL

Static acute toxicity tests were run in 3 L glass aquaria with 10 fish in each exposure container. These static toxicity tests were run using JP-4 layered directly onto the water surface. (These were defined as being "neat" exposures as opposed to using the WSF of the JP-4.) These toxicity tests were run to determine the effect of a solitary, one time spill of JP-4 jet fuel in a static pond-type of environment where very little mixing would occur. A series of concentrations of JP-4, from 1 to 100 parts per thousand (ppt), was used for rangefinding toxicity tests. The actual static tests were run with 1, 2, 3, 5, 8 and 10 ppt. They were conducted as 96-h tests with observations for lethality made at 15 min, 0.5, 1, 6 and every 24 h thereafter. The pH and dissolved oxygen in each tank were also determined daily.

Dynamic 96-h flow-through toxicity tests were conducted using a modified Mount-Brungs dilutor system made of glass and teflon (Figure 3). These studies were performed to model what might happen in a situation where there was a continual discharge of jet fuel in an environmental system, such as a stream, where mixing would occur. This was considered the most realistic way to evaluate lethality as

mixing would occur, at least from wind action, in most natural environments. To avoid WSF loss due to excess head space, toxicant chambers were sized and covered to reduce air space. Diluent and WSF mixing occurred in a closed system using glass Y tubes. The dynamic test was repeated three times with 10 fish per each of six concentrations. Observations of fish lethality, pH and dissolved oxygen were made on the same time basis discussed above for the static tests. The results were analyzed with log-probit (Finney, 1976) and Spearman-Karber (Hamilton, et al., 1977) procedures.

2.4 VENTILATORY RATE STUDIES

Ventilatory studies were conducted on bluegill using a system developed by Cairns and coworkers (e.g. Cairns and Thompson, 1980; Thompson, et. al., 1983). Bluegill (SL approximately 5.0 cm) were randomly placed in small plexiglass chambers (700 ml) with front and rear electrodes (Figure 4). After placement in the chambers fish were allowed to acclimate for 2 d before background ventilatory rates were determined. Approximately half the fish were subsequently exposed to WSF JP-4; the others served as

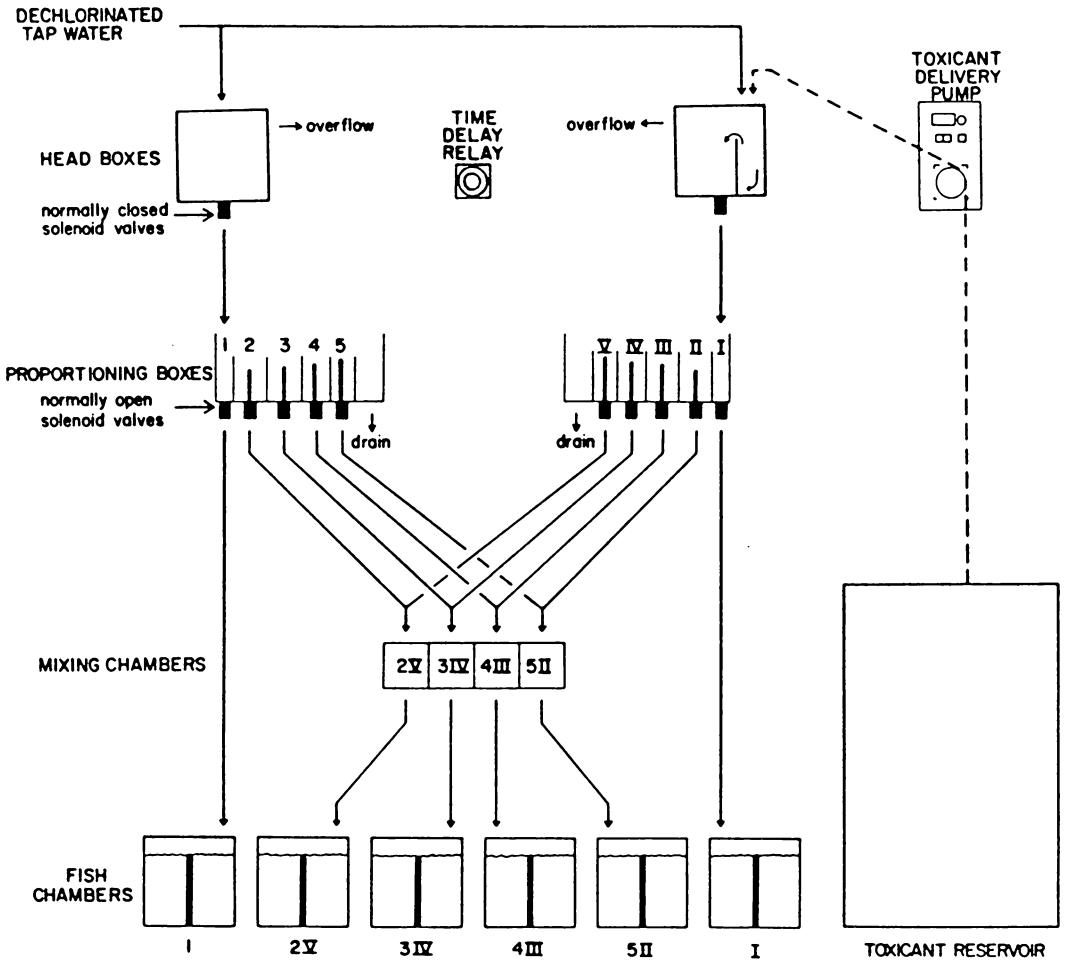


Figure 3: Dynamic diluter system used for acute toxicity studies.

controls, and ventilatory data were collected for another 24 h. The fish chambers were located in a closed container with constant light from three-15 watt bulbs. The container was buffered from vibration in the laboratory by anchoring its supports in sand; eliminating much extraneous stimuli (Figure 5). Fish movement was detected as a small voltage change (approximately 50 mv) by the electrodes; this was then amplified and converted from analog to digital format by existing hardware and software for a Digital Equipment Corp PDP8/E computer (Thompson et al., 1978). A ventilatory count was defined as any electric pulse that had both a positive and a negative peak in excess of a threshold of one volt after amplification. Gruber, et al. (1979) have shown that each of the pulses corresponds to one full ventilatory cycle of a fish. The computer assisted program counted ventilatory rates and averaged the amplitudes of opercular beats over 15 min periods. This information was then stored on a magnetic tape by a Decassette TU60 cassette tape drive and printed by a Decwriter II (LA36).

After data collection was completed, the minicomputer was tied into the Virginia Polytechnic Institute and State University's (VPI&SU) main frame computer system running VM/CMS on a IBM370-1580. The data were then transferred

directly to the Conversational Monitoring System (CMS) for later statistical analysis. The data were analyzed with ANOVA or Wilcoxon rank sum procedures if the Kolmogorov D statistic showed data to not be normally distributed.

2.5 PREFERENCE/AVOIDANCE STUDIES

Fish were given a side-by-side challenge of clean (carbon dechlorinated tap water) and WSF JP-4 influenced water in preference/avoidance studies; a system slightly modified from that of Lubinski et al. (1977) was used (Figure 6). The exposure chamber was contained within an isolation box and fish behavior was observed via closed circuit TV so that fish were subjected to as little extraneous stimuli as possible. The concentration of WSF JP-4 was determined on samples collected from ports at the bottom of each side of the chamber.

Three different observational techniques were used to determine fish behavior in the preference/avoidance studies:

1) Fish were visually observed directly on a closed circuit TV system. Five fish were placed in the exposure chamber for each study and their positions manually recorded every 30 sec.

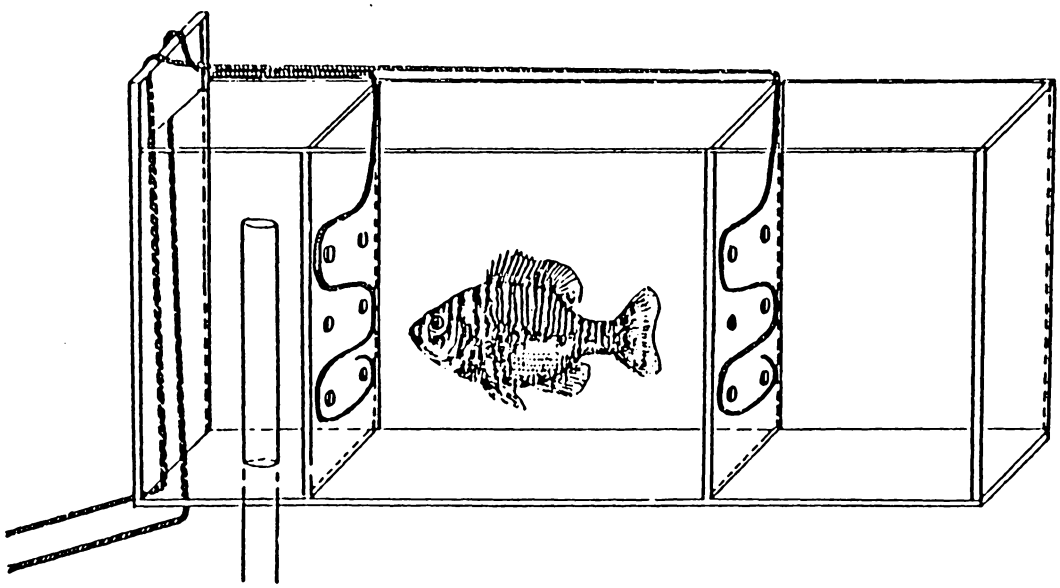


Figure 4: Detail of experimental chamber used to measure ventilation of bluegill.

2) On alternate 30 sec periods, the number of fish movements was visually quantified by dividing the TV screen into quadrants (Figure 7). A movement was defined as any time any fish crossed any quadrant line.

3) Measurements of time fish spent on either side of the tank, dilution water only side vs WSF influenced side, were also made every 30 sec using a programed Cromenco Z-2 computer (an 8080-based microcomputer). All fish were seen by the system as blocks of dark "pixels" on the light TV screen. The number of blocks of dark pixels for each quadrant were totaled every 30 sec. This information was then stored on floppy discs for later retrieval and transfer to VPI&SU's IBM 370-1580 for statistical analysis. This technique was used to confirm the results obtained visually; visual observations were used for all data reported in Section 3.4.

Fish were removed from the holding tanks and placed in the preference/avoidance chamber to acclimate for 1 to 2 h prior to exposure to WSF JP-4. Two separate 6 h control runs in which no WSF JP-4 was added were carried out to verify that there was no preference by fish for any section of the exposure chamber. In the first exposure study, three 10 min observations of behavior were made at control (0%

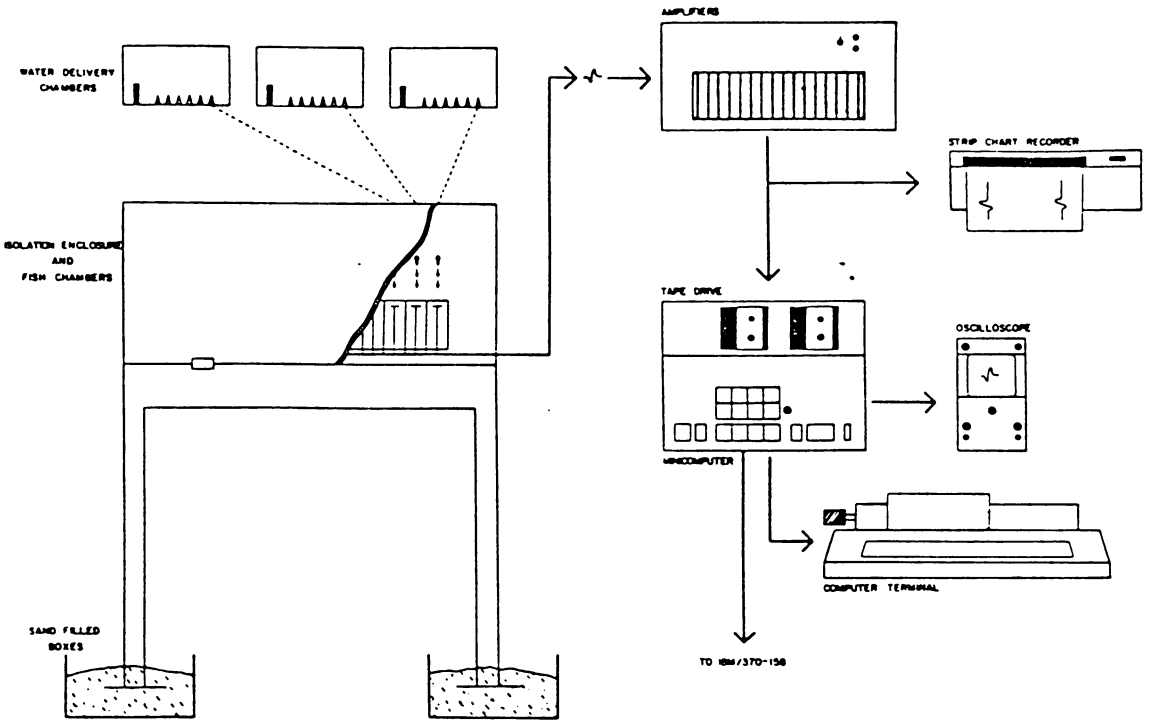


Figure 5: Experimental setup for ventilatory studies.

WSF) level. WSF JP-4 was then pumped to one side and after allowing 5 min for the concentration to come to steady state, behavioral observations were again made. After taking a sample from the exposure side, the concentration of WSF JP-4 was increased to the next higher level. This study was repeated eight times, alternating the exposure side for each study; it is shown as Study 1 in Figure 8.

After results of the first study indicated possible acclimation of fish to WSF JP-4, another study was conducted in which the concentration of WSF JP-4 was increased directly from 0% WSF JP-4 to the highest level tested in Study 1. This study was conducted twice, alternating the exposure side; it is shown as Study 2 in Figure 8.

In the last study, fish were exposed to more rapid increases, and higher final concentrations of WSF JP-4. Due to requirements for high flow rates only one 7-min observation of behavior was made at each concentration in each replicated study. This study was repeated 8 times, again alternating exposure sides, and is shown as Study 3 in Figure 8.

The above data were analyzed using ANOVA to determine if there were any significant differences. If differences were found an LSD procedure was used to determine where the differences were.

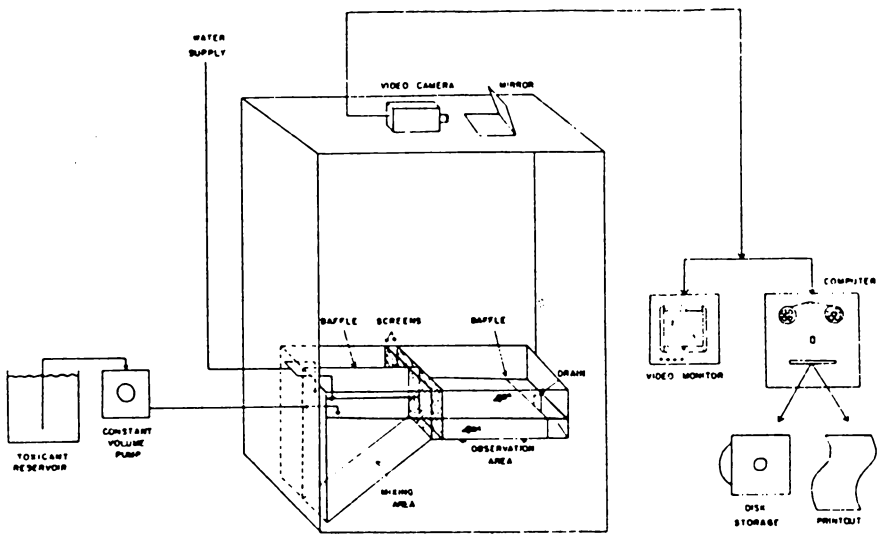


Figure 6: Experimental equipment for preference-avoidance studies with bluegill.

2.6 BLOOD CHEMISTRY

Fish were exposed to two different concentrations of WSF JP-4, 13% for 96 h and 26% for 24 h, to evaluate their effect on blood chemistry parameters. These exposures were approximately 50 and 100% of the 96-h LC50. Exposed and control fish were bled after anesthetization in a 5% benzocaine bath. The caudal peduncle was excised and blood removed from the dorsal aorta using 1 cc needleless syringes. When possible, determinations were performed on blood from individual fish. In most cases blood from two or more fish was pooled into one sample in order to obtain sufficient volume to perform all desired analyses.

The blood parameters studied were divided into two groups: whole and serum blood chemistry tests. The whole blood tests consisted of hematocrit, hemoglobin concentration and red blood cell (RBC) counts:

1) The hematocrit test, yielding percent total blood cells, e.g. RBC, white cells, platelets, etc., in whole blood, was performed using standard heparin coated microhematocrit tubes. A tube was filled with blood directly from each sacrificed fish and percent hematocrit

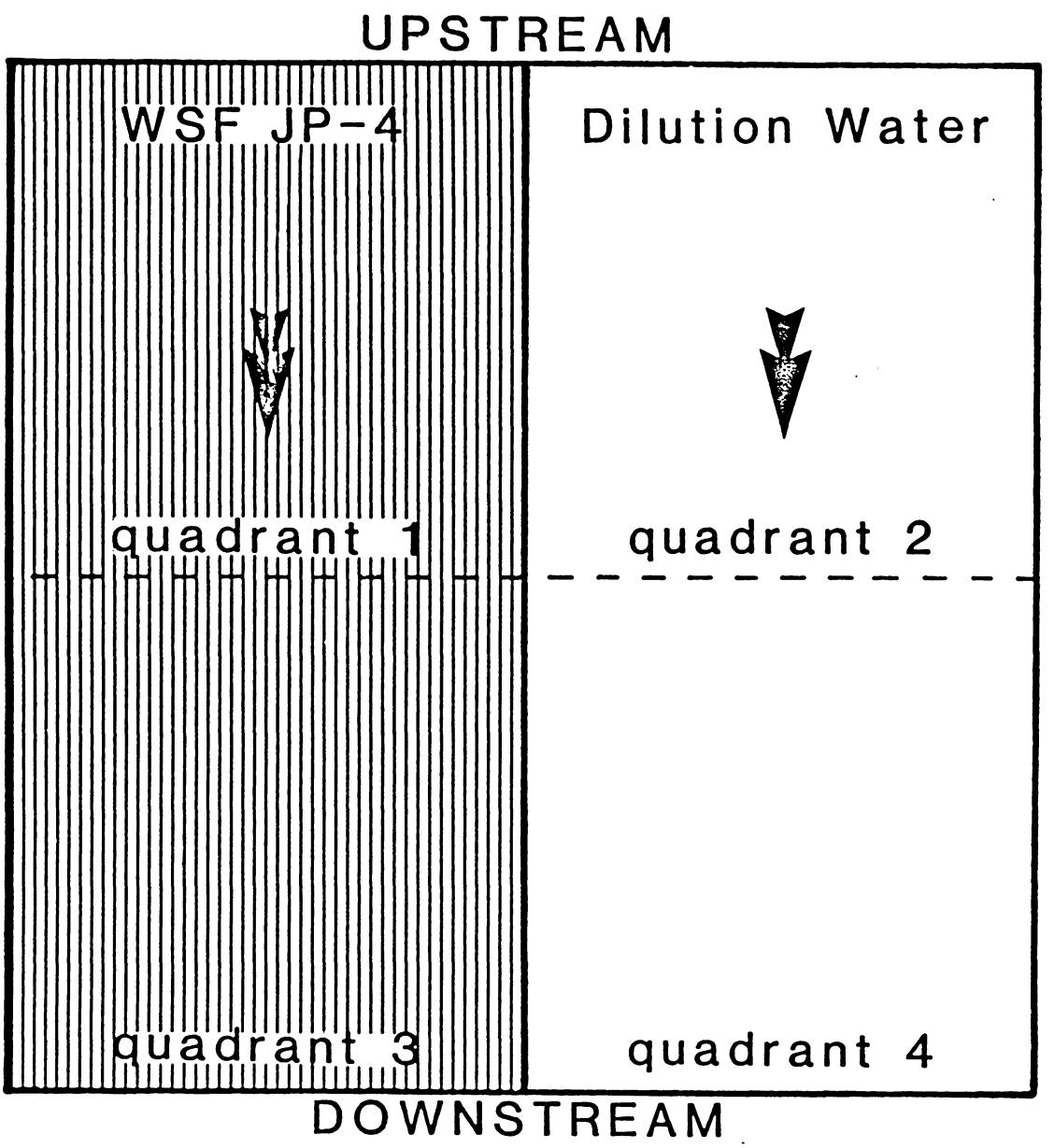


Figure 7: Example of quadrant grid for monitoring movement of bluegill exposed to WSF JP-4.

was determined with a Clay-Adams Hematocrit Reader after the tubes were spun down in a Clay-Adams Hematocrit Centrifuge for 10 mins (Wintrobe, 1974).

2) The RBC counts were conducted by diluting whole blood 1:200 with Dacie's solution and placing a small volume of diluted blood on a hemacytometer. For each count, five ruled areas on the hemacytometer were enumerated, averaged and corrected to cells mm^{3-1} (Hessler, 1960). The morphology of the RBCs was also checked at this time.

3) Hemoglobin concentration, an indicator of oxygen carrying capacity, was measured on pooled samples of whole blood using the cyanmethemoglobin method (Wintrobe, 1974).

Blood serum was analyzed using a Gilford 3500 computer assisted blood chemistry analyzer, applicable Gilford reagents and appropriate technical instructions. Blood serum tests were conducted for:

1) Enzymes lactate dehydrogenase (LDH) (EC 1.1.1.27) and aspartate aminotransferase (SGOT) (EC 2.6.1.1);

2) Inorganic substances calcium, chloride, magnesium, and inorganic phosphorous;

3) Albumin and total protein concentration; and

4) Glucose.

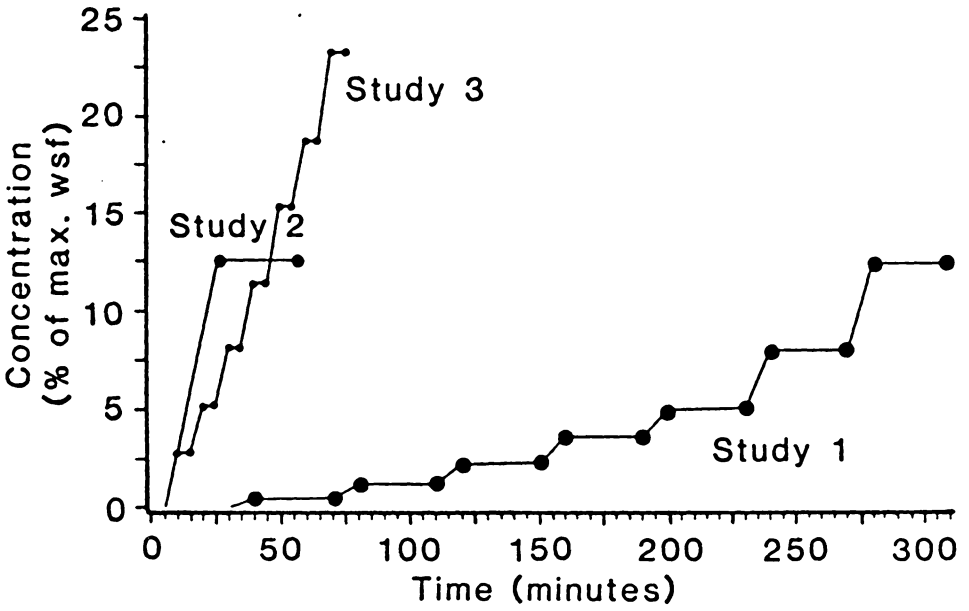


Figure 8: Time-dose exposures used in preference-avoidance studies.

Several other techniques were also used to physiologically examine the condition of fish:

1) Two different types of commercially available reagent strips for human urinalysis were used to examine fish mucous components:

a) Hemastix[1] were used to evaluate the amount of hemolysis that had taken place in fish blood (Smith and Ramos, 1976). If significant hemolysis of RBCs took place as a result of exposure to WSF JP-4 there should be detectable amounts of breakdown components in the mucous.

b) Ketostix[1] evaluated ketone bodies present in mucous of fish (Ramos and Smith, 1978). If fish were starving and breaking down fatty acids for subsequent metabolism there should be ketone bodies present in the blood and these should also appear in the mucous.

2) Tissue water content, an indicator of possible osmoregulatory problems, was measured on muscle and liver which were dissected from fish, weighed, dried and reweighed.

3) ATP levels, which can be an indication of sublethal stress (Kennicut, 1980; Suresh et al. 1983; Heath, 1984), in fish liver were determined with an enzymatic technique in

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[1] Registered trademark of Miles Laboratory, Inc.

which an ATP reaction with phosphoglycerate kinase was observed with a spectrophotometer (Jaworek et. al. 1974).

The data were analyzed using a Wilcoxon rank sum procedure to determine significant differences as the data were not uniformally normally distributed.

2.7 HISTOLOGY OF FISH

The same fish that were exposed in the blood chemistry studies discussed above were used for the histology studies. Liver and gill tissues were removed from fish immediately after they had been bled. The tissues were fixed initially with gluteraldehyde and then with osmium tetroxide (Meek, 1976). After fixation, tissues were dehydrated with a series of increasing concentrations of alcohol:water dilutions, ending with a 100% alcohol solution. After dehydration tissues were embedded in Spur's embedding media and then shaped and sectioned on a microtome. Thick sections (approx. 50 um) were cut for viewing with a Leitz Dialux light microscope with attached camera. Thin sections (approx. 5 um) were cut for viewing with a JOEL-JEM 100C electron microscope.

Comparative studies were made of tissue from control and exposed fish using photomicrographs taken of both tissue sections. In addition to visual impressions of the general state of the tissues, electron micrographs of liver tissue were evaluated for morphological differences with a planitmetry technique modified from that of Weibel (1969). Random micrographs of tissue were taken and relative amounts of different cellular components were calculated and statistically compared. The electron micrographs were taken at 33,000X and centered to insure that there was a nucleus and at least one cell in the final electron micrograph. The actual planitmetry method consisted of making 8X10 prints of the micrographs and overlaying a sheet of graph paper over each print. The intersection of the major graph lines were then punched with a sharp point so that each electron micrograph was impressed with a grid of 54 holes (6 columns of 9 holes) spaced 2.54 cm apart. A number of electron micrographs were punched at the same time and since the graph paper was opaque, the distribution of the holes on each electron micrograph was random; there was no a priori placement of the grid. Each hole was considered as a positive count if it landed on one of the tissue organelles of interest such as a vacuole, endoplasmic reticulum, mitochondria, a nucleus or glycogen deposits. The number of

positive counts were then compared using a t-test to determine if there was any significant difference in the relative counts of the different cellular materials.

III

RESULTS AND DISCUSSION

3.1 GENERATION AND ANALYSIS OF WSF JP-4

The fractionator efficiently generated a reliably uniform concentration of WSF JP-4. The analysis of WSF JP-4 showed the two primary components, benzene and toluene, to be present in MaxWSF at 16.4 ppm and 12.2 ppm respectively. These values agreed very well with those provided by the source of the JP-4, the USAF Fuels Laboratory, Wright-Patterson AFB, Ohio. A complete chemical characterization was not attempted; one can be found in Smith et. al. (1981).

One problem encountered with the fractionator was the growth of a microorganism capable of very rapid degradation of the soluble components of the WSF JP-4 during the longer exposure periods. This was controlled by periodic dissembling of the apparatus, disinfection with chlorine and washing with soap and water followed by copious rinsing with dechlorinated water.

3.2 TOXICITY OF JP-4 JET FUEL

The static toxicity test of the "neat" JP-4, using a log-probit analysis (Finney, 1976), resulted in a 96-h LC50 of 1.74 parts per thousand (ppt) (95% Fiducial Limits (FL) = 1.27 to 2.13 ppt; slope = 7.5±2.13; X^2 = 0.72). Although this is reported as a 96-h result, realistically it was only a 24-h test due to the high volatility of the JP-4 components. Within the first 24 h period the concentrations of benzene and toluene were reduced by >50%.

The individual dynamic acute toxicity tests resulted in few partial kills so that the log-probit statistical analyses could not be performed on each repetition. A Spearman-Karber statistical analysis (Hamilton et al., 1977), which does not depend on number of partial kills was used to generate a LC50 for the dynamic toxicity tests (Rice et. al., 1977). With this statistical technique, the calculated 96-h LC50 was 26.2% (95% FL = 24.8 to 27.7%) MaxWSF. This is approximately equal to 11.6 ppm of total soluble JP-4 components (Fisher et al. 1984). In order to estimate the slope of the toxicity curve, data from all replicates of the dynamic toxicity tests were combined for a log-probit analysis. The result was a 96-h LC50 of 28.8% WSF (95% FL = 26.0 to 33.4%; slope = 14.02±7.92; X^2 = 0.87)

(These two 96-h LC50s were not significantly different as determined by overlapping confidence intervals.) The advantage of performing a log-probit analysis on all data even though it is not a normally accepted procedure was that it illustrated the steepness of the dose-response curve. Ninety-nine percent of lethality occurred between 19.5 and 38.8% WSF JP-4 (Figure 9).

The 24-h (25.5% WSF), 48-h (26.4% WSF) and 72-h (28.7% WSF) LC50's were also determined using log-probit analysis of available data for each time period. Relative potentcy analysis showed that there was no significant difference between these four values (C.L. = 95%)(Finney, 1976). Apparently WSF JP-4 is toxic to fish based on a threshold of toxicity rather than on increased time of exposure. There were few significant deaths of fish in the toxicity tests after the first 24 h. Although there were great losses of the more volatile components within 24 h, the 96-h LC50 reported by Fisher et. al. (1984) for a similar fuel had an equivalent slope (9.9 to 12.8) and a higher LC50, 42 to 48% WSF JP-4, which would be expected for a static test.

The acute toxicities of the main components of WSF JP-4 to bluegill have been reported by other investigators. Benzene was found to have a 96-h LC50 of 22.49 mg/L (Pickering and Henderson, 1966). In their review, Buikema

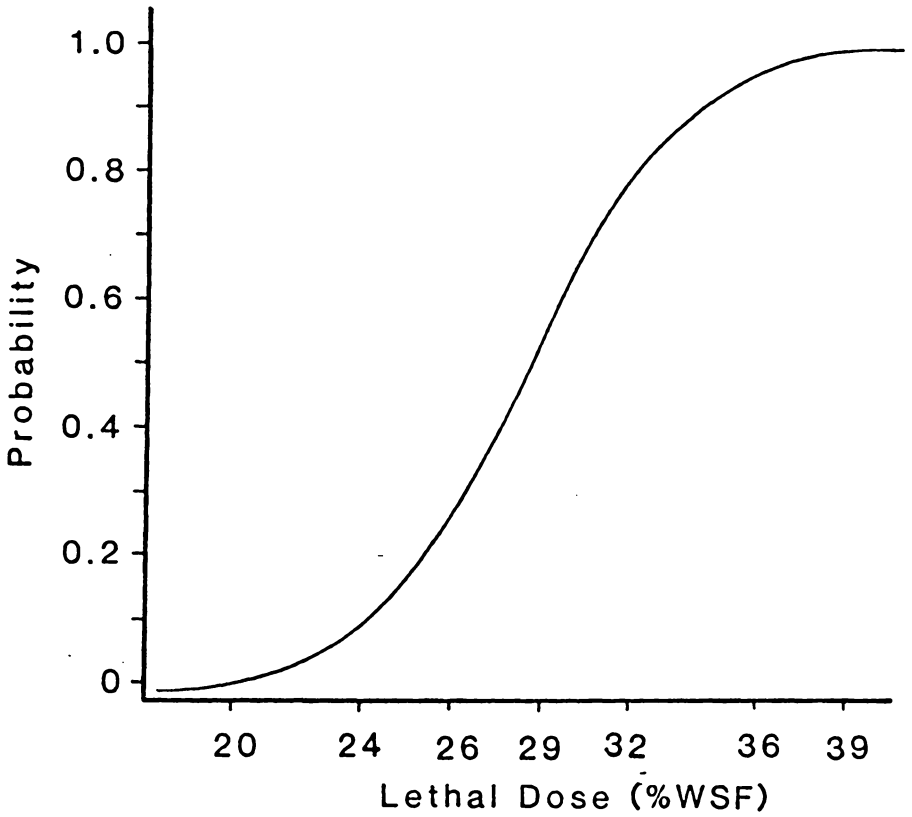


Figure 9: Dose-response acute toxicity curve for WSF JP-4.

and Hendricks (1980) quote values for the 24-h LC50 of 20 and 32 to 34 mg/L for benzene. The reported 96-h LC50 for toluene was 24.00 mg/L (Pickering and Henderson, 1966) and 6.41 to 8.01 mg/L (Korn et al. 1979). The 24-h LC50's quoted by Buikema and Hendricks (1980) were 18.9 and 24 mg/L. As WSF JP-4 is a composite of benzene, toluene and other minor components, the LC50s for the major components would make it appear that they act additively with the less concentrated components of WSF JP-4 in exerting their toxic effect. A calculated 96-h LC50 for WSF JP-4 from the above data would be approximately 18 ppm. Since this calculation is based on static data it compares well with the dynamic data for WSF JP-4 (LC50 = 11.6 ppm), which would be expected to show a higher toxicity. This also supports the assertion that benzene and toluene are the more toxic components of petroleum products (Craddock, 1978; Morrow, 1975). The data for WSF JP-4 is also comparable to that presented by Thomas and Rice (1979) for the WSF's of Cook Inlet Crude (LC50 = 1.73 ppm) and No. 2 fuel oil (LC50 = 0.651 ppm) for pink salmon fry.

3.3 VENTILATORY STUDY

There was a significant linear relationship between ventilatory rates and concentration of WSF JP-4 ($p < 0.01$, $r^2 = 0.72$, Figure 10). A comparison of the change in ventilatory rate compared to change in concentration showed the same general trend ($p < 0.01$, $r^2 = 0.71$, Figure 11).

A preliminary statistical analysis compared ventilatory rates for groups of exposed fish to those for control fish as blocks using ANOVA procedures. This is similar to procedures used by other investigators (e.g. Bloem, 1983). The results of this statistical analysis are shown in Table 1. The threshold for a shift in ventilatory rate was determined to be approximately 15% WSF JP-4 based on this statistical analysis procedure. However, individual variability masked the differences that were seen in the same fish between control and exposure periods.

A second statistical analysis treated fish individually by comparing median ventilatory rate for the last 4 h of control data to the first 6 h of exposure data for each fish after deleting 2 h of exposure data to allow time for WSF JP-4 to completely replace the contents of each exposure chamber. A statistically significant diurnal variation in ventilatory rates of control fish was noted so a different

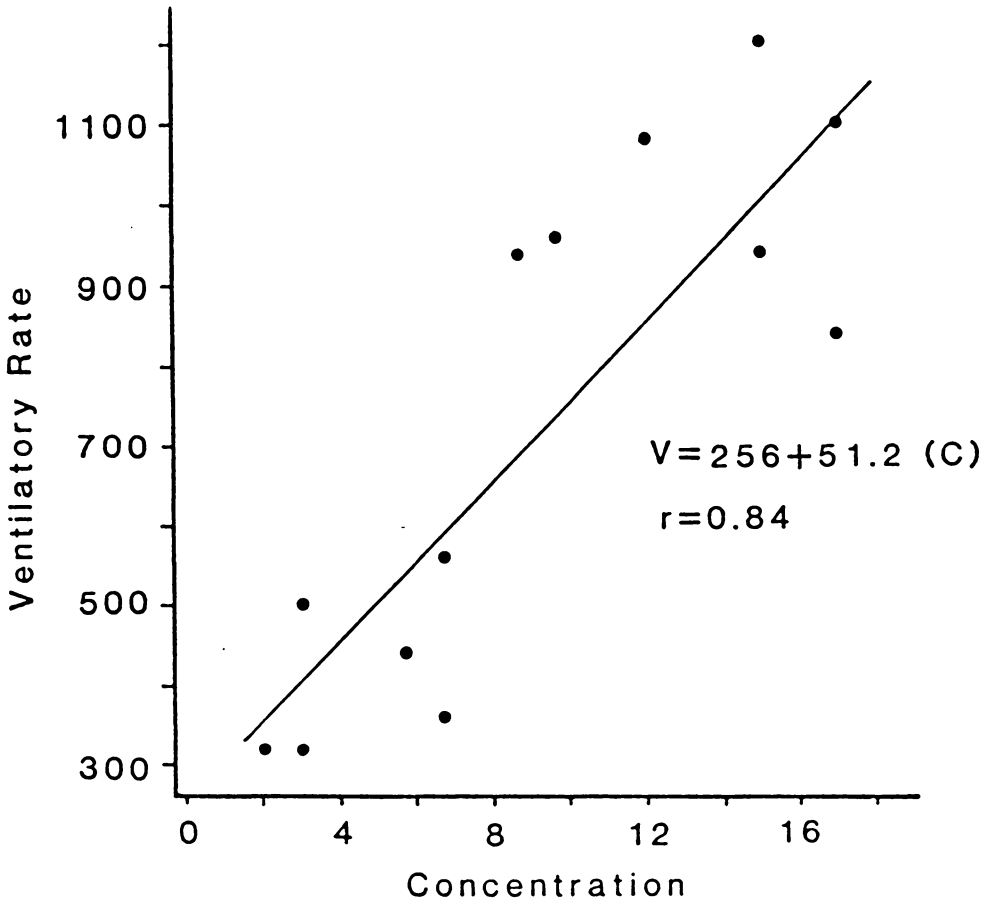


Figure 10: Ventilatory rate as a function of WSF JP-4 concentration.

strategy was used to analyze the data. Four h of data for exposed fish were compared to control data collected for each individual fish during the same 4 h period of the previous day (Figure 12).

These data were not always normally distributed in contrast to those of Nunn et. al. (1982). Data for some fish had the null hypothesis of normality rejected at a p = 0.05 level of confidence. While the skewness (1.022) and kurtosis (0.395) of the distribution showed little appreciable deviation from normality, a conservative nonparametric paired rank sum procedure was used to compare differences in median ventilatory rate before and after exposure (Hollander and Wolfe, 1973). This analysis was performed on all data. Results of this statistical analysis on ventilatory data are in Table 2. The 24 h studies were more valid.

Using a p = 0.05 level of confidence as a cutoff, the 5.1% WSF JP-4 concentration was determined to be the level at which a "threshold" shift in ventilatory rate could be detected. This was approximately 20% of the 96-h LC50 and less then the 96-h LC01. If same day data were considered, a similar trend was seen although there was more "noise" in this treatment. Changes in amplitude of ventilation

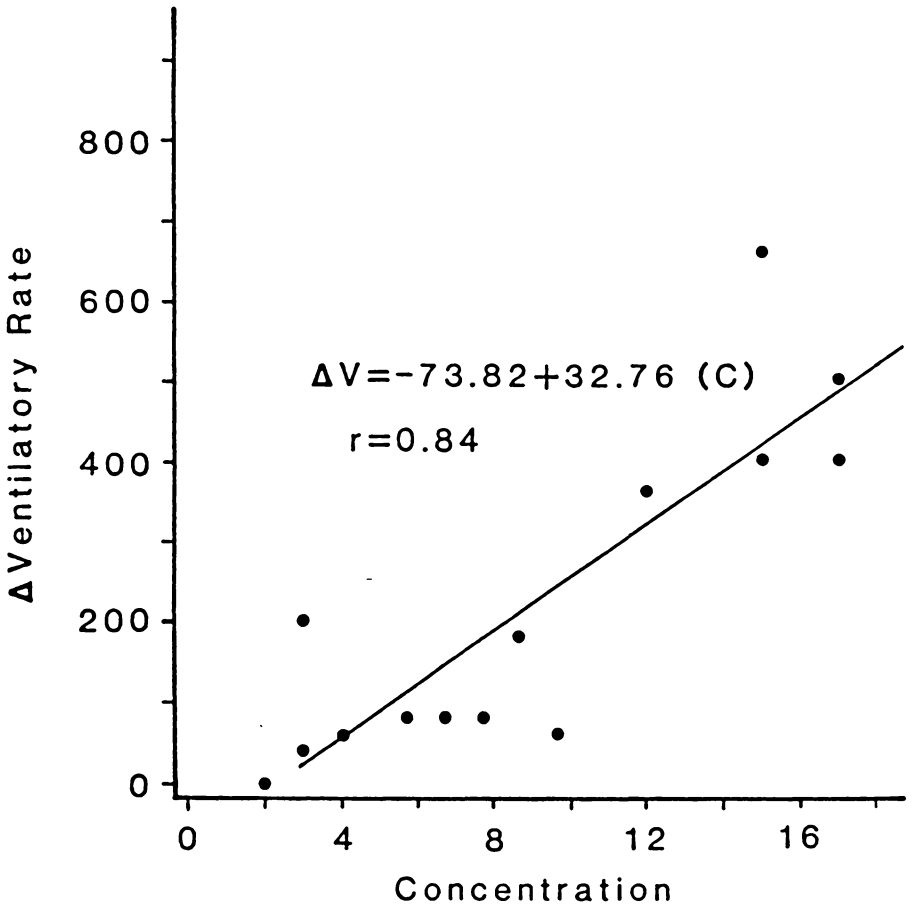


Figure 11: Ventilatory rate change as a function of WSF JP-4 concentration.

TABLE 1

Probability values for ventilatory rate change in bluegill exposed to WSF JP-4 (ANOVA)

Concentration WSF JP-4 (%)	n	p-values[1] Exposed	Control
8.5	8	0.67	0.14
10	5	0.10	0.50
12	4	0.10	0.99
14	5	0.14	0.95
15	8	0.01	0.60
17	3	0.02	0.70

[1] Significance level = $p < 0.05$

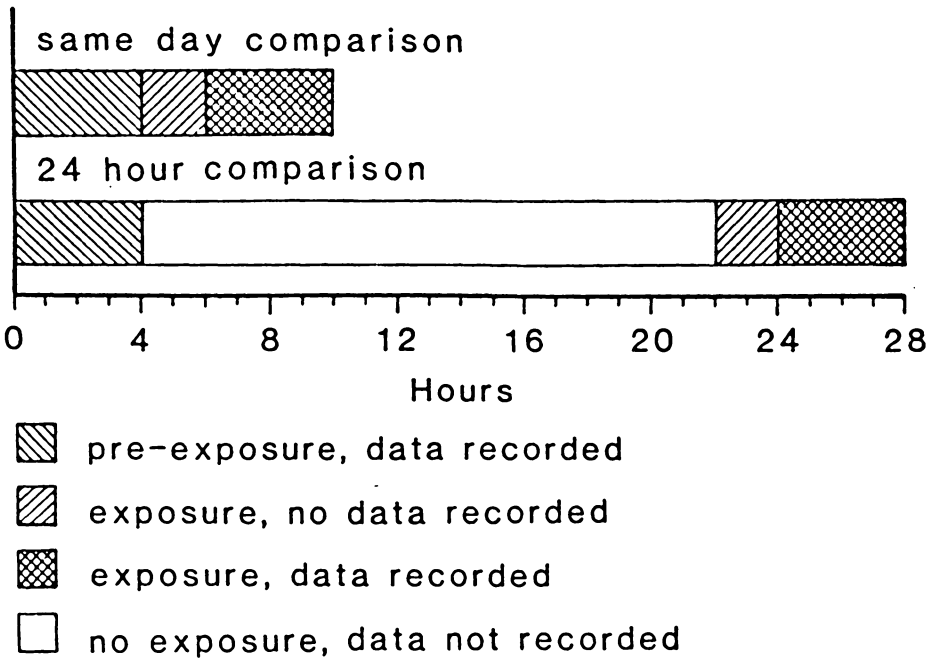


Figure 12: Display of comparative time periods used to analyze ventilatory data.

TABLE 2

Probability values for ventilatory rate comparisons of bluegill exposed to WSF JP-4.

Conc of WSF JP-4[1]	Number of fish	Exposed[2]		Control	
		Vent.	Amp.	Vent.	Amp.
2.1	9	0.36	0.22	-	-
2.8	7	0.15	0.22	0.25	0.25
3.1	7	0.11	0.29	0.11	0.34
5.1	7	<0.05	0.02	-	-
6.7	9	<0.01	0.06	0.23	0.50
8.5	8	<0.01	<0.01	0.23	0.16
12.0	4	0.06	0.19	0.50	0.34
15.0	13	<0.01	<0.01	0.02	0.29
17.0	8	<0.01	<0.01	<0.01	0.45

[1] This is % of maximum soluble amount of JP-4 in water.
[2] Significance level = $p < 0.05$.

responded similarly with a significant shift also occurring at 5.1% WSF JP-4 (Table 2).

There seemed to be a second plateau or threshold of sensitivity in ventilatory behavior at 15 and 17% WSF JP-4. The actual p-values were an order of magnitude lower than those for 5.1 to 12% WSF JP-4. The control fish at these higher concentrations also had significantly elevated ventilatory rates. There was no obvious reason for this shift in the ventilatory rate in the control fish, i.e., a change in experimental procedure or other external causes. It is hypothesized that this was a "sympathy" response to the more agitated behavior of the exposed fish. As the chambers were partially transparent the fish could observe the changes in behavior of other fish. The concentration of this secondary threshold correlated well with the ANOVA test discussed above (Table 2) that demonstrated a significant effect when the fish were analyzed as groups. It is possible that there was a change in effect on the fish, i.e., that a different physiological effect is taking place at the higher WSF JP-4 concentration.

The earlier ventilatory studies of Thomas and Rice (1979) also showed an increase in ventilatory rate when salmon were exposed to petroleum hydrocarbons, but they used much higher relative concentrations, approximately 80% of LC50s, of Cook

Inlet crude and No. 2 fuel oils. Brocksen and Bailey (1973) reported increased ventilatory rates for salmon and striped bass exposed to benzene. It is possible that the increased ventilatory rate in this study was the result of an increased metabolic rate that could be partially due to the stress and also to the actual metabolic detoxification of the ingested and absorbed petroleum components.

3.4 PREFERENCE/AVOIDANCE STUDIES

There were two > 5 h replicates in which fish were not exposed to any WSF JP-4; these were controls for fish behavior over time. There was no significant difference in the side of the exposure chamber that fish chose over time ($p = 0.62$ for "exposure side"). There was also no significant change in the number of movements over time in these control runs ($p = 0.29$, Figure 13).

In the first study, fish were exposed to dilutions of up to 12% WSF JP-4. In this study fish spent significantly different amounts of time on the exposed side of the chamber ($p < 0.01$, Figure 14). There was no difference in movement of fish at different concentrations ($p = 0.21$). When avoidance reactions at different exposures were analyzed

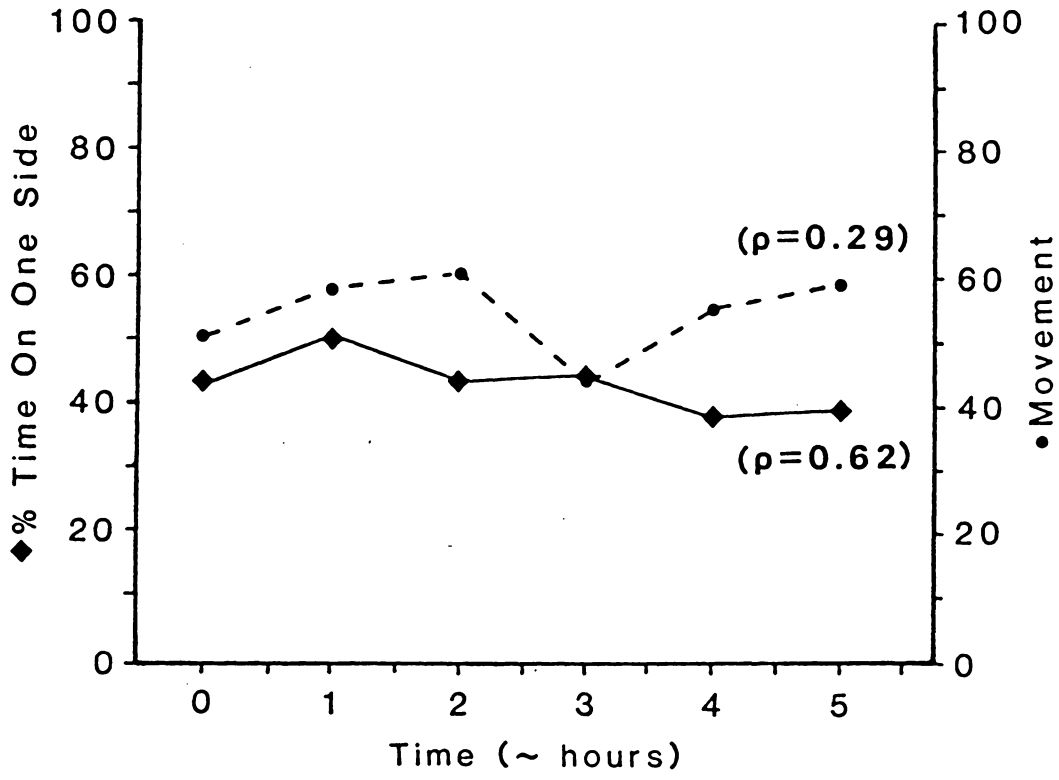


Figure 13: Control data for bluegill preference-avoidance studies.

with a LSD procedure it was determined that significantly less time ($p < 0.05$) was spent on the JP-4 influenced side when fish were exposed to 3.5% and 4.9% WSF JP-4 as compared to controls (0% WSF JP-4), lower (0.4, 1.3 and 2.2% WSF JP-4) and higher (12.2% WSF JP-4) concentrations. Fish showed no difference in behavior at the highest concentration when compared to controls. There are at least two possible explanations for this observation. First, that fish's olfactory organs were rapidly desensitized physiologically to WSF JP-4. Such a hypothesis has been advanced by Norton et al. (1982) and Gardner (1978). Second, fish became acclimated to WSF JP-4 as the level slowly increased in the preference/avoidance exposures. In order to test for this possibility the next preference/avoidance study was conducted.

In the second study fish were exposed to the control, 0% WSF JP-4, and then to the highest level of exposure from Study 1, 12% WSF JP-4. If acclimation affected fish behavior in Study 1, it was hypothesized that they would actively avoid sudden exposure to a high concentration of WSF JP-4. Fish showed no significant difference in avoidance or movement behavior when exposed to 12% WSF JP-4 (Figure 15).

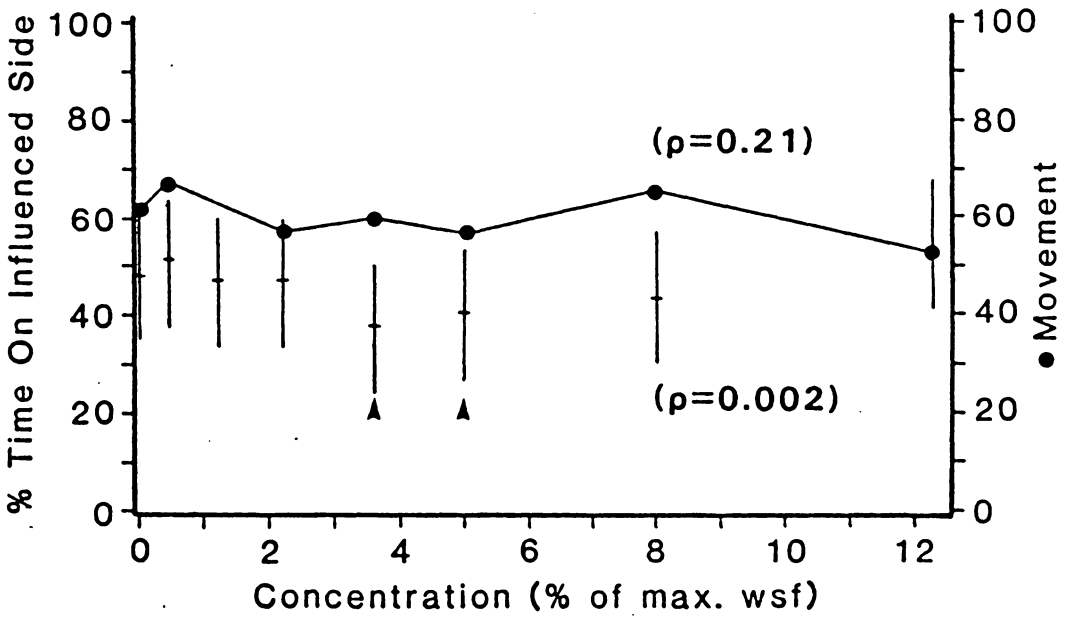


Figure 14: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 1.

The third study was conducted with more rapidly increasing and higher concentrations of WSF JP-4 than Study 1 in order to determine fish behavior when exposure level approached the 96-h LC50. There was no significant difference in either avoidance behavior or movement ($p = 0.08$ and 0.06, respectively; Figure 16). This may partially be due to the rapidity of increasing concentration and high level of "noise" in fish behavior due to a fewer number of observations. It should be noted that these p-values were close to a 0.05 significance level and avoidance behavior at median levels of concentration, 3.7% and 5.2% WSF JP-4, was distinctly greater than for control and high (23.3% WSF) concentrations. Study 3 results confirmed those of Study 1 although they were not as significant.

Although difficult to quantify, there was an apparent difference in general behavior of fish during these studies. When fish were first placed in the chamber they seemed to swim randomly with little or no schooling behavior. When WSF JP-4 reached the median levels at which the avoidance behavior was manifested, there appeared to be a more noticeable attempt by fish to escape the preference/avoidance arena entirely (Figure 17). The more normal random swimming pattern is shown on the left and the

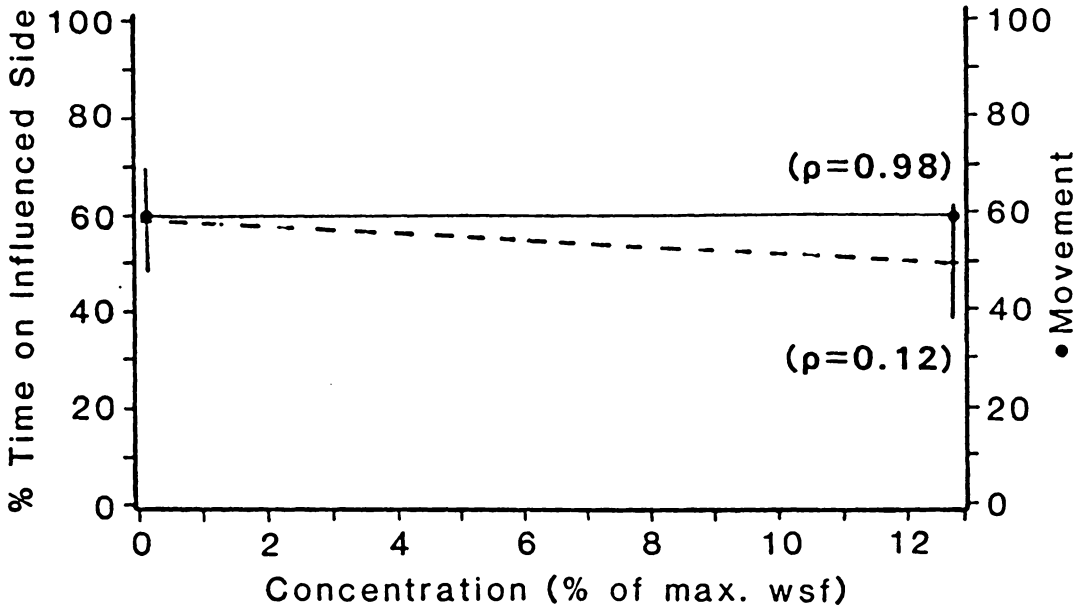


Figure 15: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 2.

"escape behavior" is shown on the right. This behavior was discernable with most groups of exposed fish.

Juvenile salmon have been shown to avoid 1.9 to 3.7 mg/L total petroleum hydrocarbons (Maynard and Weber, 1981). Adult salmon have been shown to avoid moderate to high concentrations of petroleum hydrocarbons in a fish ladder choice study in the natural environment (Weber et al., 1981). Other workers have reported fish not avoiding petroleum contamination in a marine environment that was so severe that the water was cloudy with suspended petroleum. In related studies, Folmar et al. (1981) noted decreased predation by coho salmon; this could be attributed to failure of olfaction. Many species of fish have been shown to depend heavily on olfaction for feeding (Kleerekoper, 1969).

3.5 BLOOD CHEMISTRY/PHYSIOLOGY

A data base for "normal" physiology was collected before the fish were exposed to WSF JP-4. These data are presented in Table 3. Most of these parameters were not normally distributed. This problem was discussed by Miller et al.

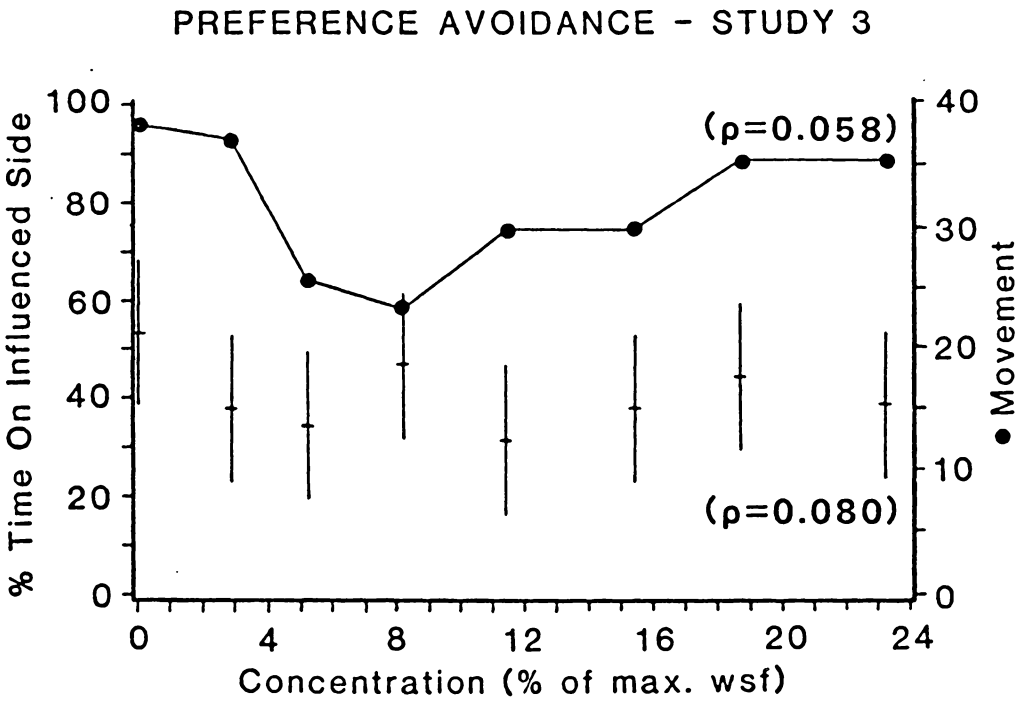
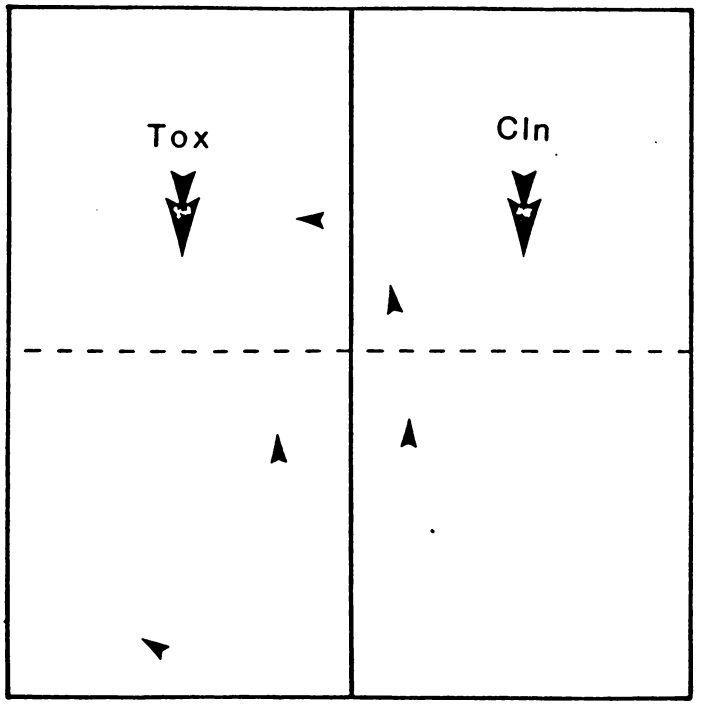


Figure 16: Effects of WSF JP-4 on preference-avoidance behavior of bluegill: Study 3.

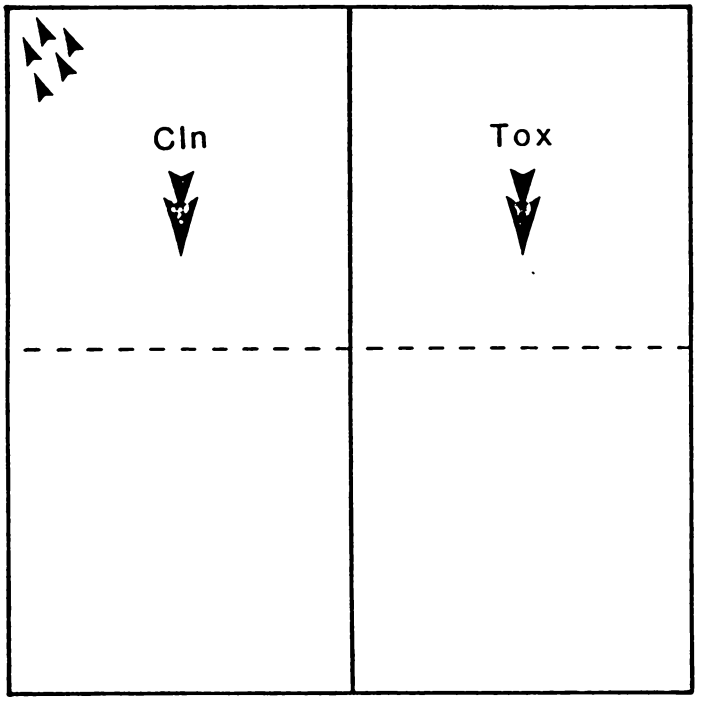
(1983). Because of non-normality, the data were reanalyzed with a nonparametric procedure (Brunden et al. 1970). (The same Wilcoxon rank sum nonparametric procedure used for analysis of the ventilatory data was used.) In Table 4 the median values and 95% confidence limits are presented for the different physiological parameters.

Fish were initially exposed to 13% WSF JP-4, half of the nominal 96-h LC50. This concentration was equal to the 96-h LC04 based on the log-probit curve but was originally chosen on the basis of the initial ANOVA block analysis discussed above which showed that this concentration was the threshold for a shift in ventilatory rate (Table 1). As this exposure was for only 96 h it was not likely that many physiological changes would be seen. A second study was conducted with an exposure concentration which approximated the 96-h LC50 of 26% WSF JP-4. This study should detect changes that could be expected in fish just before the concentration became lethal. This second exposure lasted for 24 h due to the anticipated high mortality of exposed fish.

At the higher concentration the expected kill of approximately 50% of the adult fish occurred. This confirmed that the previous lethality determinations, based on experiments with juvenile fish, were applicable to adult



A: Normal behavior of fish in preference/avoidance chamber.



B: "Escape" behavior of fish in preference/avoidance chamber.

Figure 17: "Escape behavior" of bluegill at moderate levels of WSF JP-4.

TABLE 3

Physiological parameters of control bluegill.

Parameter	n	Mean	S	CV	S^2	Prob:D	99%	1%	Range
Size									
Length	80	4.77	0.59	12.4	0.35	<0.01	6.80	2.70	4.10
Weight	79	58.0	28.0	49.0	800.	<0.01	179.0	31.5	151.0
Mucous									
Ketosis	33	0	-	-	-	-	-	-	-
Hemolysis	33	5.85	2.73	46.7	7.40	<0.01	9	0	9
Muscle									
Water content-flesh (%)	33	74.9	0.007	0.92	<0.0001	<0.01	75.7	72.7	3.0
Blood									
Hemoglobin (g/100ml)	72	7.0	1.1	16.3	1.3	0.13	9.5	4.4	5.1
Hematocrit (%)	80	38.6	6.6	17.1	43.7	>0.15	53.9	25.0	28.9
RBC ($\times 10^6/mm^3$)	58	1.59	31	20	1000	>0.15	239	98	151
Calcium (mg/dl)	67	23.4	14.3	61	204	<0.01	99	8.5	90.5
Chloride (mg/dl)	68	138	88	63	7746	<0.01	516	33	482
Magnesium (mg/dl)	61	4.3	1.9	44.1	3.6	<0.01	13.9	2.4	11.5
Phosphorous (mg/dl)	69	28.7	13.2	45.9	173.5	<0.01	81.0	13.8	67.2
Total protein (g/dl)	78	5.0	1.6	31.7	2.6	<0.01	9.4	2.7	6.7
Albumin (g/dl)	75	1.77	0.54	30.27	0.29	<0.01	4.4	0.85	3.55
LDH (mg/l)	73	2.884	0.15	0.54	2400	0.06	8.106	0.323	7.783
SGOT (ug/l)	60	.524	166	0.32	27.78	0.02	259	0.259	0.774
Glucose (mg/dl)	24	71.9	19.0	26.4	360	<0.01	128	43.6	84.4
Liver									
Water content (%)	33	77.0	0.04	5.02	0.002	0.28	71.8	82.9	11.1
ATP (umoles/ml)	30	3.22	0.62	20.6	0.38	0.03	4.81	2.12	2.69

TABLE 4

Nonparametric analysis of control fish blood parameters.

Parameter	n	Median	95% Confidence Interval Lower Limit	Higher Limit
Size				
Length	33	8.0	4.5	8.0
Mucous				
Ketosis	33	0	-	-
Hemolysis	33	7.00	6.33	8.00
Muscle				
Water content-flesh (%)	33	75.2	74.7	75.4
Blood				
Hemoglobin (g/100ml)	72	7.1	6.9	7.4
Hematocrit (%)	80	38.0	36.5	40.5
RBC ($\times 10^6/mm^3$)	58	1.49	1.60	1.66
Calcium (mg/dl)	67	18.4	14.7	25.2
Chloride (mg/dl)	68	102.2	98.5	110.0
Magnesium (mg/dl)	61	4.0	3.8	4.3
Phosphorous (mg/dl)	69	25.2	21.7	30.0
Total protein (g/dl)	78	4.60	4.30	5.1
Albumin (g/dl)	75	1.60	1.50	1.80
LDH (mg/l)	73	2.553	2.016	3.390
SGOT (ug/l)	60	.506	0.416	0.577
Glucose (mg/dl)	24	69.0	62.8	73.6
Liver				
Water content (%)	33	70.0	69.6	71.0
ATP (umoles/ml)	18	3.22	2.60	3.78

bluegills. Although no narcosis of fish was seen at the sublethal concentrations used for preference/avoidance studies, there did seem to be such an effect in this study. Just before fish expired they were suspended head up/tail down in the water column. This behavior was also seen in the acute toxicity tests before death of test fish.

Control fish were subjected to the same procedures as above except they were not exposed to WSF JP-4 during the observations. When the two sets of control fish were compared there were significant differences between them (Table 5). There were no extrinsic reasons for these differences. The fish were collected at the same time from the same source, had been similarly treated, and showed no signs of disease. The tests were run in close time proximity so no effects due to seasonality were suspected. Possibly the differences were due to the different lengths of holding time under test conditions.

Because the control fish were significantly different from each other the exposed fish for each study were compared to their own respective group of controls (Table 6). (A comparison that was made between the exposed fish for each study and the data base for all nonexposed fish yielded the same final results that are discussed below, so the differences between the sets of control fish did not

affect the final outcome of the entire study, it only confounded the interpretation of the significance of physiological data.) The physiological results for these two exposure studies are in Table 6. The measured values are presented as median values with p-values generated by the nonparametric Wilcoxon rank sum procedure.

The 13% WSF JP-4 resulted in no significant differences between control and exposed fish for the "whole body" parameters; hemolysis, ketosis and percent water content of muscle (Table 6). At the higher concentration, 26% WSF JP-4, there was a significantly greater percent water content in exposed fish muscle and no change in hemolysis or ketosis. This would suggest that fish exposed to near lethal concentrations have osmoregulatory problems.

For the three whole blood component measurements; hemoglobin, hematocrit and red blood cell counts, there were no significant differences observed in fish exposed to the lower WSF JP-4 concentration. At the higher concentration there were significant decreases in all three parameters. The most likely explanation for this difference is that there was a blood dilution effect due to the osmoregulatory problem mentioned above.

There was a significant decrease in chloride ion concentration but not in the other three serum ions measured

TABLE 5

Comparison of control fish data for two exposure studies.

Parameter	Control 13% WSF JP-4	Control 26% WSF JP-4	p-value
Size			
Length	4.4	4.4	0.42
Weight	45.7	41.8	0.92
Mucous			
Ketosis	0	0	-
Hemolysis	5.0	6.8	0.05
Muscle			
Water content-flesh (%)	75.0	76.0	0.82
Blood			
Hemoglobin (g/100ml)	8.1	7.0	<0.01
Hematocrit (%)	46.3	40.6	<0.01
RBC ($\times 10^6/mm^3$)	1.37	1.54	0.18
Calcium (mg/dl)	38.4	30.0	<0.01
Chloride (mg/dl)	101.6	93.6	0.26
Magnesium (mg/dl)	4.2	4.1	0.40
Phosphorous (mg/dl)	30.0	24.6	0.06
Total protein (g/dl)	7.0	6.2	0.01
Albumin (g/dl)	2.4	2.0	<0.01
LDH (mg/l)	5.000	3.826	0.04
SGOT (mg/l)	0.483	0.510	0.96
Glucose (mg/dl)	66.8	70.5	0.22
Liver			
Water content (%)	75.0	70.0	<0.01
ATP (umoles/ml)	3.86	2.57	0.02

TABLE 6

Physiological and blood parameters in bluegill exposed to WSF JP-4.

	13% WSF JP-4			26% WSF JP-4		
Parameter	Control	Exposed	p-value	Control	Exposed	p-value
Mucous						
Ketosis	0	0.3	0.39	0	0	-
Hemolysis	5.0	5.5	0.76	6.8	6.5	0.41
Muscle						
Water content (%)	75.3	75.3	0.14	76.1	77.3	0.01
Blood						
Hemoglobin (g/100ml)	8.1	8.2	0.71	7.0	4.0	0.01
Hematocrit (%)	46.3	44.4	0.67	40.6	33.8	0.04
Red blood cells ($\times 10^6/mm^3$)	1.37	1.70	0.23	1.48	0.98	<0.01
Calcium (mg/dl)	38.4	41.3	0.82	30.0	28.0	0.44
Chloride (mg/dl)	101.6	83.4	0.05	93.6	78.4	<0.01
Magnesium (mg/dl)	4.5	5.0	0.50	4.1	4.0	0.75
Phosphorous (mg/dl)	30.0	34.2	0.17	24.6	29.4	0.04
Total protein (g/dl)	7.0	7.0	0.08	6.2	5.8	0.20
Albumin (g/dl)	2.4	2.6	0.21	2.0	1.8	0.22
Lactate dehydrogenase (mg/l)	5.000	4.914	0.71	3.826	5.153	0.21
Aspartate aminotransferase (ug/l)	0.482	0.601	0.17	0.510	1.053	<0.01
Glucose (mg/dl)	66.8	91.4	0.01	70.5	439.	<0.01
Liver						
Water content (%)	69.8	71.0	0.05	70.0	77.5	<0.01
ATP (umoles/ml)	3.86	2.90	0.01	3.86	1.31	0.01

in fish exposed to the lower WSF JP-4 concentration. At the higher WSF JP-4 concentration blood chloride ion concentrations were significantly lower; calcium and magnesium were not significantly changed; and phosphorous was significantly elevated. The decrease in chloride ion concentration for both exposure concentrations would again indicate an osmoregulatory problem.

There were no significant shifts in serum enzymes LDH and SGOT for fish exposed to the lower WSF JP-4 concentration; nor were there any changes in total protein or albumin levels. (Glucose was the only serum parameter which was significantly elevated ($p = 0.01$)). At the higher WSF JP-4 concentration there was no shift in the level of serum LDH, but the levels of both glucose and SGOT were significantly elevated. These were changes that could not be attributed to a suspected osmoregulatory problem in exposed fish. It is hypothesized that there was metabolism of accumulated JP-4 components in liver. There were sequestered materials that stained positive for lipids visible in the microscopic examination of liver. Other authors have noted the storage of conjugated and nonconjugated metabolic byproducts of toxicants (Roubal, et. al., 1977 and 1978). This metabolism could require the increased levels of transaminase (SGOT) that were seen. More likely, the elevated SGOT levels could

be the result of liver damage from the exposure to WSF JP-4. This is the clinical significance of increased SGOT in humans (Ravel,1969). Elevated LDH might also be expected in liver damage, but such elevations are reported to be much less sensitive indicators and slower to respond (Zilva and Pannall, 1971).

Although there were no differences in levels of total protein or albumin, there was a pronounced increase in the level of glucose in blood of all exposed fish. This may be the result of metabolism as mentioned above or simply due to fish mobilizing their stores of glycogen. There did appear to be fewer glycogen deposits present in the liver of fish after exposure to WSF JP-4. This was particularly noticable after the longer exposure periods used for the 13% WSF JP-4. This loss of glycogen and elevated glucose have been reported by Heitz et al. (1974) in livers of mullet exposed to Empire Mix crude oil.

There were significant differences at both concentrations for effects on liver. Both exposure levels resulted in increased water content of liver. This could be the result of osmoregulatory problems and perhaps of changes in liver tissue because of sequestering of metabolites. There was also significant decrease in the amount of ATP present in the liver of fish exposed to both concentrations. These

lower ATP levels may be just a reflection of the higher water content of the liver. Similar results were seen by Heath (1984) when fish were exposed to sublethal levels of copper.

Overall, WSF JP-4 appears to affect osmoregulation and liver function. These effects were much more pronounced in fish exposed to the higher, 26%, WSF JP-4 concentration. Similar results were reported by Heitz et al. (1974) who studied liver enzymes in salmon exposed to Empire Crude Oil for 4 d. They also found increased levels of liver glucose which were attributed to carbohydrate metabolism. The depletion of glycogen stores was also reported by Hawkes (1979) after rainbow trout were exposed to Prudhoe Bay crude oil.

Similar osmotic imbalances were measured as changes in chloride levels after hydrocarbon exposure and were attributed to an alteration in cell membrane permeability in the gills (Morrow et al., 1975).

3.6 HISTOLOGY OF FISH

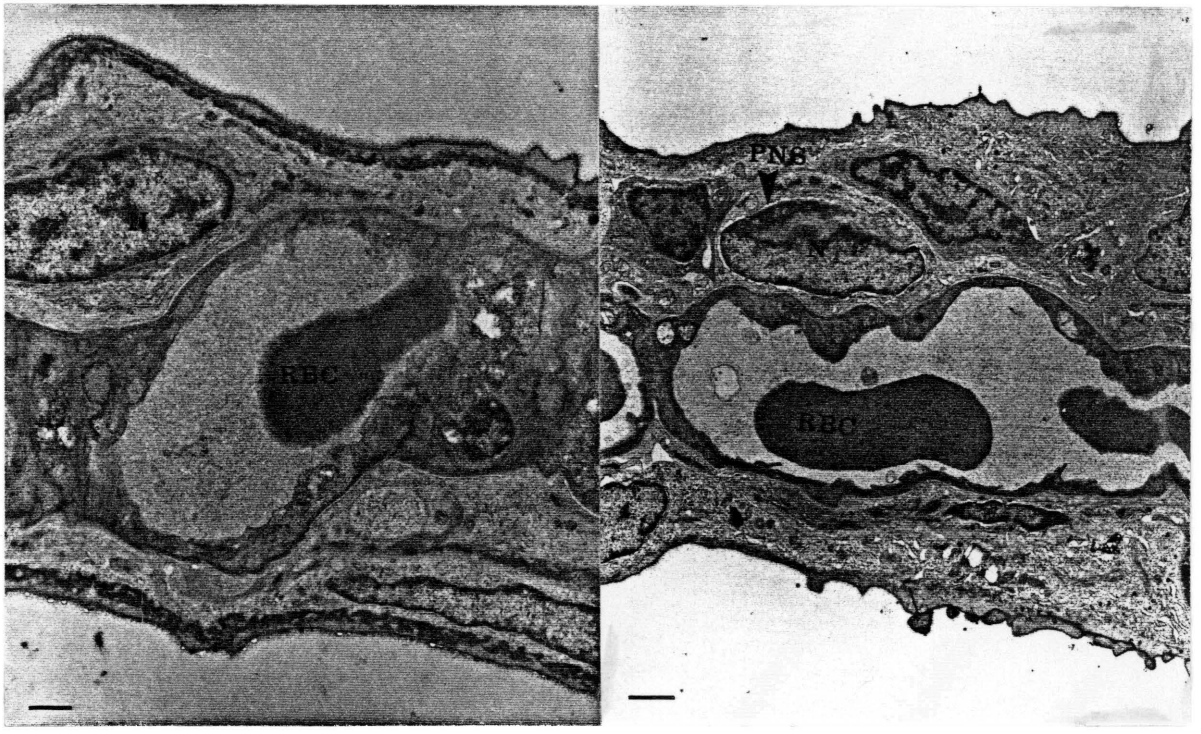
The lower WSF JP-4 concentration (13%) produced no effects upon gill tissue that were discernable when the tissues were examined by light or electron microscopy. At

the higher WSF JP-4 concentration the only observed effect was an extended paranuclear space in many cells (Figure 18). Other investigators have reported hyperplasia (DiMichele and Taylor, 1978; Gardner, 1978) as well as cellular lesions and edema (Norton, 1981) in the gills of fish exposed to petroleum hydrocarbons. The lack of these effects in gill tissue after exposure to WSF JP-4 can be partially explained by the fact that at this higher concentration the exposure period was only for 24 h. This might not have been a sufficient time for notable tissue changes. Solangi (1980) stated that many histological changes were not manifested until after several days (10-15) of exposure. This would seem to be especially true in looking for such effects as hyperplasia where new cellular material must be generated. Preliminary investigations with longer, near lethal exposures, had resulted in an increase in ruthenium red staining mucopolysaccharide materials in the gill (Doane, unpublished). Similar results were reported by Norton (1981). The mucous cells in the gills of fish exposed to the high concentration in this study appeared to be normal and there appeared to be no abnormal buildup of mucous (Figure 19).

The livers of fish exposed to both concentrations of WSF JP-4 were more pale and of different texture than those from

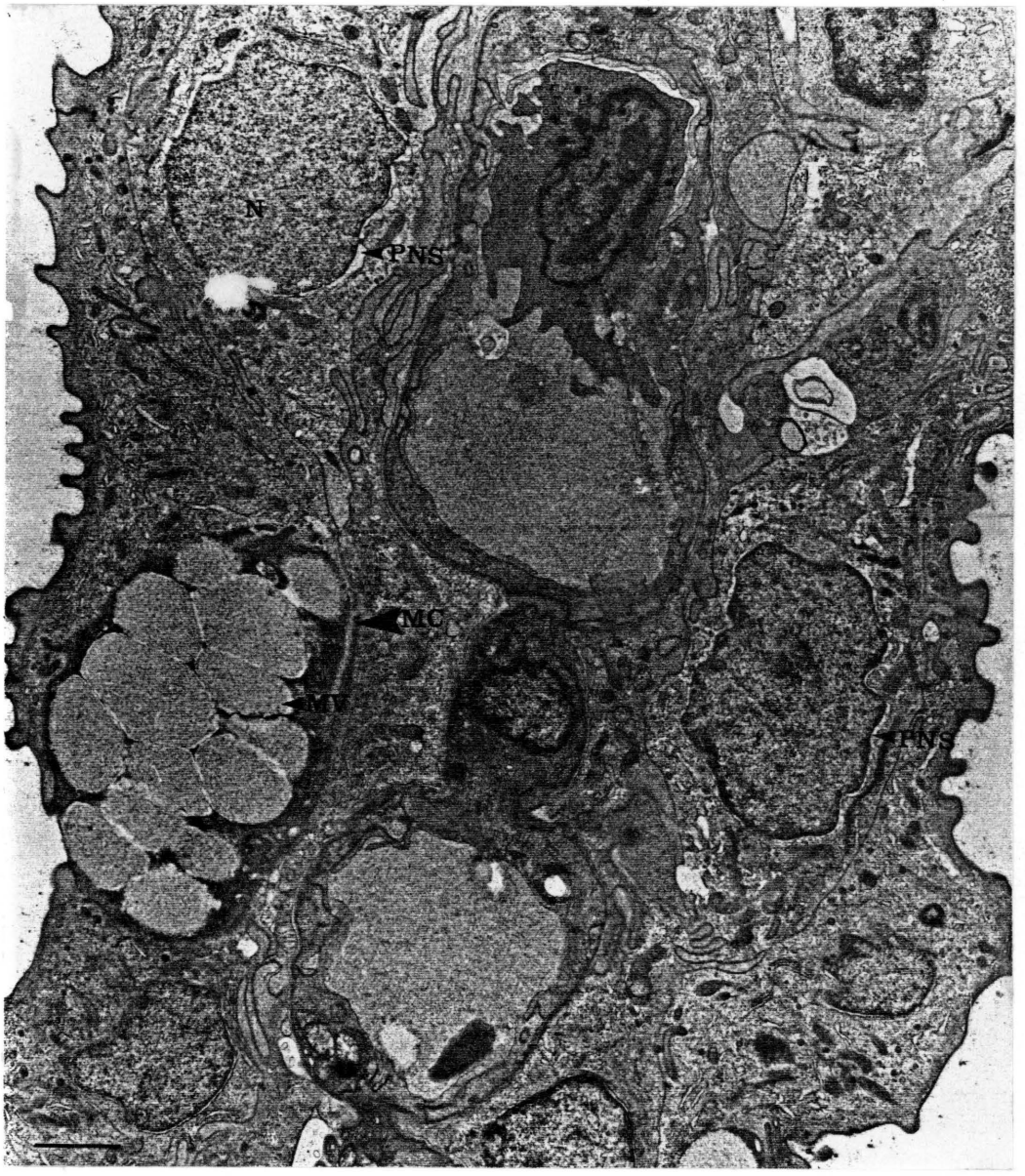
A: Control gill

B: Exposed gill



N = Nucleus
PNS = Paranuclear space
RBC = Red blood cell

Figure 18: Electron micrographs of gill tissue from control and exposed fish.



MC = Mucous cell
MV = Cytoplasmic mucous vacuole
N = Nucleus
PNS= Paranuclear space

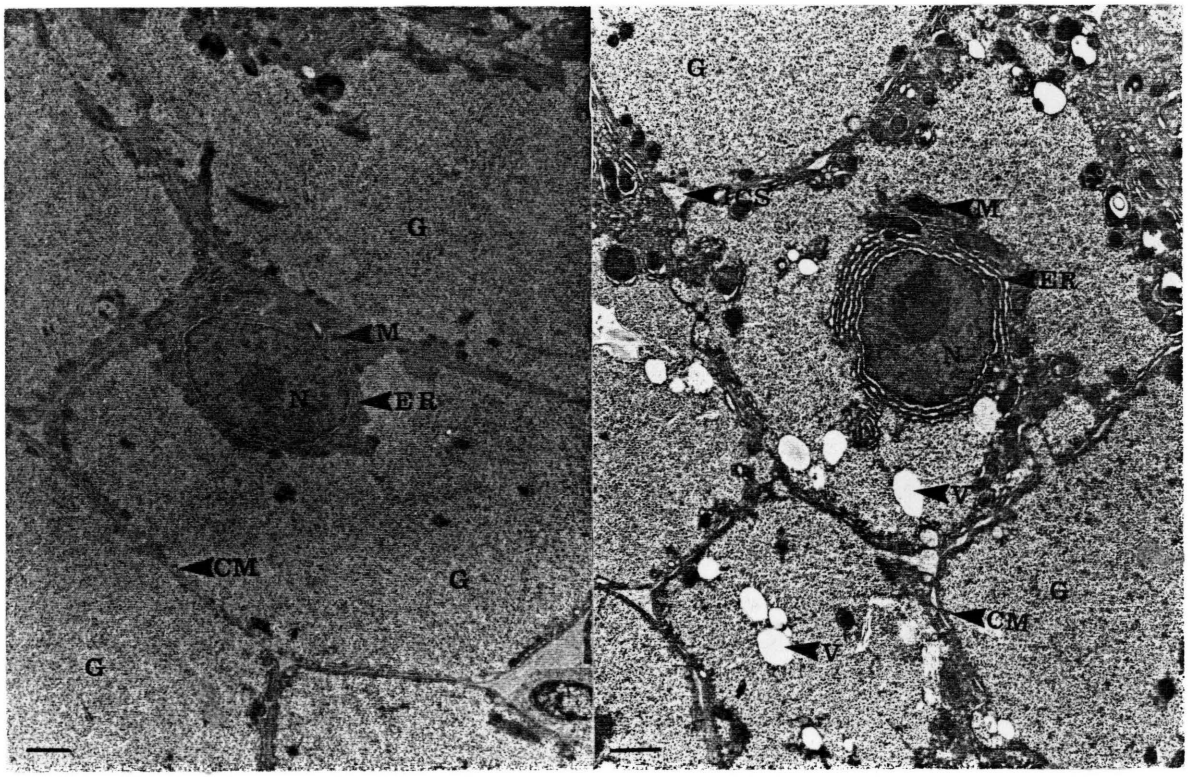
Figure 19: Electron micrograph of mucous cell from gill of fish exposed to 26% WSF JP-4

control fish. At 100x light magnification there were many small lipid staining vacuoles in the hepatocytes of livers of fish exposed to both concentrations. These were assumed to contain sequestered metabolites of JP-4. There were very few similar vacuoles in the hepatocytes of livers from control fish. There have been reports of such stored metabolic products after exposure to petroleum hydrocarbons (Roubal, 1975; Solangi, 1980) There was also a discernable reduction in amounts of stored glycogen in the livers of fish exposed to the lower concentration, but not in those exposed to the higher concentrations of WSF JP-4. As mentioned above, this correlated well with increases in glucose levels. Similar findings were reported by Hawkes (1979) and Solangi (1980). These lipid vacuoles were also visible in the electron micrographs of exposed liver cells (Figure 20). There was also a greater separation between individual liver cells in the exposed fish. These spaces appeared electron dense and were suspected of also having sequestered JP-4 component materials. Although other investigators have reported changes in the general amount of endoplasmic reticulum in liver cells exposed to petroleum hydrocarbons (Hawkes, 1979) this did not seem to be the case in the livers of fish exposed to WSF JP-4. In an effort to

substantiate these impressions of relative changes in glycogen levels, stored metabolites, and no changes in other cell organelles, a random planimetry method (see Section 3.6) was used to obtain data that could be statistically analyzed. The results of t-tests on the relative number of positive "counts" for 9 electron micrographs of control and 11 of exposed fish livers for these cellular components are given in Table 7. As can be seen, this statistical treatment, although on a relatively few number of electron micrographs, supports the visual impressions discussed above. The category of nucleus was included as a check on the similarity of cell types between the control and exposed fish. If there was no difference in "counts" for cell nuclei, as was the case, it was hypothesized that the random electron micrographs were taken of similar cells in the respective livers. Also, although this was not apparent in these livers, Solangi (1980) had reported swollen nuclei in livers of fish exposed to Empire Mix crude oil WSF.

It should be noted that much of the literature that has been referred to for histological studies was conducted using chronic exposures extending for longer periods of time than was the case for this study. Therefore it might be expected that longer exposure periods would result in more definitive histological changes. More study would be

A: Control liver B: Exposed liver



CM = Cell membrane
ER = Endoplasmic reticulum
G = Glycogen deposits
I = Intercellular space
M = Mitochondria
N = Nucleus
V = Vacuole

Figure 20: Electron micrograph of liver tissue from control and WSF JP-4 exposed fish.

TABLE 7

Results of t-tests on quantities of cellular components of control and 26% WSF JP-4 exposed fish liver.

Components	"Counts"[1] Control	Exposed	p-values
Vacuoles	0.33	2.36	<0.01
Endoplasmic reticulum	12.45	15.45	0.18
Mitochondria	3.11	3.27	0.87
Nucleus	3.22	2.37	0.24
Glycogen	28.89	28.54	0.94

[1] "Counts" = Presence of hole punched in cellular component in electron micrograph.

required to confirm this. Anderson et al. (1981) reported that the product of time and concentration was relatively consistent for toxicity of Prudhoe Bay crude oil to shrimp, but whether this would hold up for sublethal effects to tissues is not known.

IV

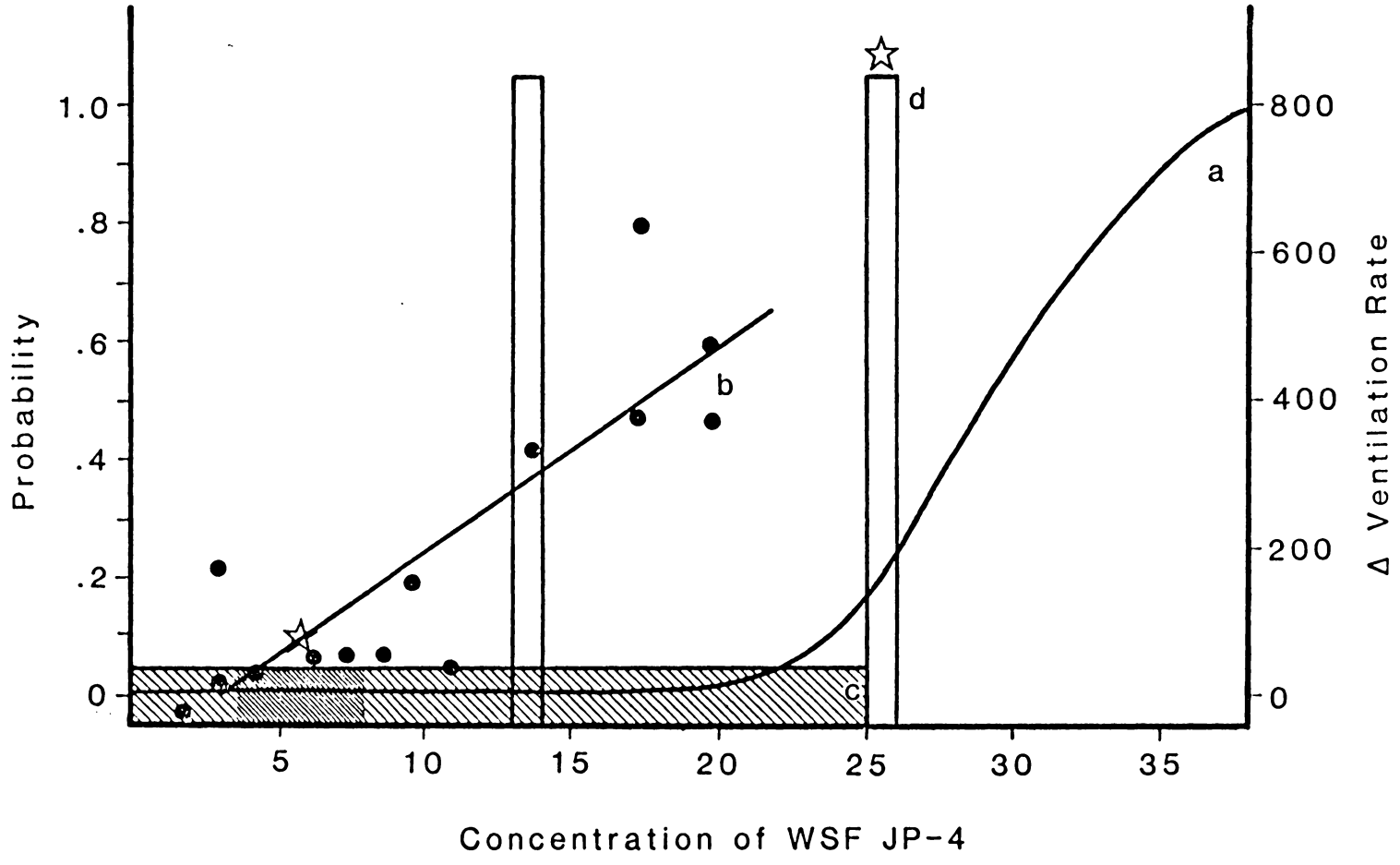
CONCLUSIONS

The interrelationships of results of the lethality, ventilatory and preference/avoidance studies on bluegill using WSF JP-4 are shown in Figure 21. This figure also shows the relative concentrations of WSF JP-4 used for the physiology/histology studies.

The 96-h LC50 for WSF JP-4 was 26.2% (95% FL = 24.8 to 27.75%) of the maximum soluble fraction (MaxWSF). The toxicity curve was very steep with 99% of the toxicity (LC01 to LC99) occurring between 19.5% and 38.8% of MaxWSF. The 24-h, 48-h, 72-h and 96-h LC50s were not significantly different when compared by relative potentcy analysis.

Increased ventilation rate in exposed bluegill sunfish was detected at a sublethal concentration, 5.1% WSF JP-4. This was equivalent to 20% of the nominal 96-h LC50 but less than the 96-h LC01. Biomonitoring of ventilatory rate of bluegill was therefore an excellent method for approximating the NOEL. There were few significant adverse physiological effects at an exposure concentration several times higher in value.

A second biomonitoring procedure, preference/avoidance behavior, proved to be a much less sensitive method of



a = lethality curve
b = ventilatory response
c = preference/avoidance response
d = physiology/histology studies

Figure 21: Comparison of WSF JP-4 effects on bluegill.

detecting sublethal levels of WSF JP-4. There was a significant increase in avoidance behavior at concentrations similar to those that caused a change in ventilatory behavior. However, this avoidance behavior was not seen at higher concentrations. There was no indication of narcosis or anesthetization of fish during the preference/avoidance studies. This would indicate that preference/avoidance behavior technique was not reliable for detecting the presence of jet fuel in water or detecting the NOEL. It was hypothesized that this was due to damage to fish olfactory tissue.

There were few significant shifts in physiological and blood parameters measured on fish exposed to a WSF concentration that was half the nominal 96-h LC50. There were several changes in physiological measurements for fish exposed to a higher concentration that was essentially the 96-h LC50. These included decreases in liver and muscle water content, blood cell counts and hemoglobin and serum chloride indicating osmoregulatory problems; increases in SGOT indicating possible liver damage; and increase in serum glucose indicating a generalized stress syndrome. These exhibited effects are those that just precede mortality. This would indicate that blood parameter analyses were not

effective means for determining sublethal toxicity in bluegill exposed to WSF JP-4 for short term exposures. Even at the LC50 most significant changes may be attributed to osmoregulatory problems.

The only pronounced effect seen in histological examination of gill and liver tissue was the sequestering of suspected metabolites of WSF JP-4 in lipid vacuoles in the liver. There were slight effects seen in increased paranuclear space in gill tissue and separation of cell membranes in liver tissue. There were no differences seen in endoplasmic reticulum, mitochondria or nuclei of liver tissue from control and exposed fish. This indicates that the use of ultramicroscopy is not an effective method for evaluating sublethal damage to organisms exposed to WSF JP-4.

These results clearly indicate that biomonitoring of bluegill exposed to WSF JP-4 using ventilatory rate changes is a very effective method and definitely a superior procedure compared to preference/avoidance testing, physiological or histological studies.

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COMPARISON OF BIOMONITORING TECHNIQUES FOR EVALUATING EFFECTS OF JET FUEL ON BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*)

by

Thomas R. Doane

(ABSTRACT)

The purpose of this study was to compare the simultaneous effects of the water soluble fraction (WSF) of JP-4, a common military and civilian jet fuel, on survival, ventilatory rate, preference-avoidance behavior, and blood enzymes and ions of the bluegill sunfish (*Lepomis macrochirus*) to determine possible interrelationships and which procedures might be most descriptive of sublethal stress. The dynamic 96-h LC50 for WSF JP-4 was 26.2% (percent of the maximum soluble amount of JP-4). A concentration of 5.1% WSF JP-4 caused a detectable shift in ventilatory functions (rate and amplitude); this concentration was equivalent to the 96-h LC01. Fish did not display a strong preference-avoidance reaction when exposed to WSF JP-4. Some avoidance occurred at 3.5% and 4.9% WSF JP-4. At concentrations near the 96-h LC50 fish appeared to lose their ability to detect WSF JP-4, indicating potential

for fish not to avoid lethal levels of WSF JP-4. Few significant changes in whole and serum blood parameters were measured in fish exposed to 13% WSF JP-4; at near lethal concentrations changes were primarily attributed to osmoregulatory failure. Therefore such changes in blood parameters would not be useful to validate or confirm exposure to WSF JP-4. There were few significant changes in gill and liver histology of fish exposed to WSF JP-4 other than an increase in size or number of lipid vacuoles in the liver of exposed fish.