The effects of pentachlorophenol on the liver and gills of cutthroat trout (Salmo clarki)
by John Kevin Morrison

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University


Abstract:
The effects of progressive concentrations of pentachlorophenol (PCP) on the liver and gills of cutthroat trout were examined both histologically and ultrastructurally. Tissues were collected from fish immediately after they became moribund and at the termination of a 96-hour acute toxicity test.

Cellular alterations in the liver at the light microscopic level included: focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and mild cellular necrosis. Alterations at the ultrastructural level included: decreased glycogen stores, occasional swelling of mitochon- dria with an increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae. The granular endoplasmic reticulum and the perinuclear cisterna were dilated. There was an apparent increase in the number of nuclear pores in hepatocytes from exposed fish. Large autophagic vacuoles were abundant in some hepatocytes from fish dying from PCP exposure. Hepatocyte projections into the space of Disse were hypertrophic, and membrane bound fragments similar to these projections appeared to slough into the sinusoidal lumen. No consistent or significant cellular alterations were observed in the gills of test or control fish.

This study suggests that both light and electron microscopy can be of value in identifying the pathologic insults caused by exposure of cutthroat trout to PCP.

# THE EFFECTS OF PENTACHLOROPHENOL ON THE LIVER AND GILLS OF CUTTHROAT TROUT (SALMO CLARKI)

by

John Kevin Morrison

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Veterinary Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

May, 1983

APPROVAL

of a thesis submitted by

John K. Morrison

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

May 26, 1983
Date

David M. Young
Chairperson, Graduate Committee

Approved for the Major Department

May 26, 1983
Date

David M. Young
Head, Major Department

Approved for the College of Graduate Studies

27 May 1983
Date

Michael Malone
Graduate Dean

## STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Director of Libraries when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature John K Morrison

Date May 26, 1983

## ACKNOWLEDGEMENTS

The author would like to express appreciation to his major advisor, Dr. David Young for his support; to Dr. Glen Epling for his introduction to the field of electron microscopy and for allowing pursual of specific interests in this field; to Mr. Charlie Smith for the introduction to the field of histology and for sharing his knowledge of fish histopathology for the past six years; to Dr. Robert V. Thurston for supplying facilities and supporting this thesis project; to Dr. Herb Smith for his support during application to the graduate program; and to Ms. Merrie Mendenhall for the graphics.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

LIST OF FIGURES--Continued

LIST OF FIGURES--Continued

## ABSTRACT

The effects of progressive concentrations of pentachlorophenol (PCP) on the liver and gills of cutthroat trout were examined both histologically and ultrastructurally. Tissues were collected from fish immediately after they became moribund and at the termination of a 96-hour acute toxicity test.

Cellular alterations in the liver at the light microscopic level included: focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and mild cellular necrosis. Alterations at the ultrastructural level included: decreased glycogen stores, occasional swelling of mitochondria with an increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae. The granular endoplasmic reticulum and the perinuclear cisterna were dilated. There was an apparent increase in the number of nuclear pores in hepatocytes from exposed fish. Large autophagic vacuoles were abundant in some hepatocytes from fish dying from PCP exposure. Hepatocyte projections into the space of Disse were hypertrophic, and membrane bound fragments similar to these projections appeared to slough into the sinusoidal lumen. No consistent or significant cellular alterations were observed in the gills of test or control fish.

This study suggests that both light and electron microscopy can be of value in identifying the pathologic insults caused by exposure of cutthroat trout to PCP.

# CHAPTER 1

## INTRODUCTION

Pentachlorophenol (PCP) is reported to be the most toxic member of the chlorophenol series. Fish kills, losses to wildlife, toxic effects in farm and domestic animals, and human fatalities have resulted from exposure to PCP (4, 7, 8, 19, 25, 33, 37, 47). Low but detectable levels of PCP have been found in river water, municipal water supplies, human food stuffs, as well as in blood, urine, and fat of nonoccupationally exposed humans (25). Toxicity studies using PCP have shown that it is extremely toxic to aquatic animals (9, 13, 36, 38). It has also been shown that fishes can concentrate PCP to levels 400 times that of an initial, nonlethal, water concentration (18, 23, 24, 26). This bioaccumulation of PCP represents a potential hazard in the food chain (14, 40).

Chlorophenols, including PCP, are environmentally and economically important. They are used as wood preservatives, fungicides, herbicides, insecticides, and bactericides. Some are degradation products of chemically similar herbicides such as 2,4-D (2,4 dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5 trichlorophenoxyacetic acid, popularly referred to as Agent Orange).

The sensitivity to lethal concentrations of PCP varies according to different species of fishes. Representative 96-hour LC 50 (median

lethal concentration) values are (Montana State University Fisheries Bioassay Laboratory [MSU/FBL], unpublished data):

Rainbow Trout --------- 0.115 mg/l
(Salmo gairdneri)

Fathead Minnow -------- 0.266 mg/l
(Pimephales promelas)

Channel Catfish ------- 0.132 mg/l
(Ictalurus punctatus)

Bluegill -------------- 0.202 mg/l
(Lepomis macrochirus)

Reported lethal values for some mammals under experimental conditions are (6, 46):

Rats (adults) ---------- 125-200 mg/kg body wt.

Calves ----------------- 140 mg/kg body wt.

Sheep ------------------ 120 mg/kg body wt.

There are few reports in the literature of studies directly relating, or attempting to correlate, histopathologic changes with PCP toxicity in aquatic animals (14, 32). Histopathological descriptions of mammalian toxicity studies involving PCP vary from rather vague descriptions such as fatty changes in the liver or cloudy swelling of hepatocytes (19, 43), to describing statistically significant alterations of nuclei and organelles using detailed morphometric techniques at the light and electron microscopic levels (16).

The primary functions of the fish gill include: respiration, absorption, excretion, and osmoregulation. Anatomically the gill lamellae, because of their vast surface area and direct exposure to the environment, can be considered primary target tissues in aquatic pollution assessment. The liver, the primary organ of detoxification,

should also receive special attention because of its key role in the partial metabolism of many xenobiotics (5, 17). Figure 1 shows the major detoxification pathways for PCP in fishes (21).

This study was designed to characterize the alterations induced by PCP in the liver and gill of cutthroat trout (Salmo clarki) under experimental conditions. A secondary objective of the study was to determine the feasibility of using histopathologic and ultrastructural evaluations in conjunction with morbidity and mortality to evaluate insults of toxic substances to aquatic animals.

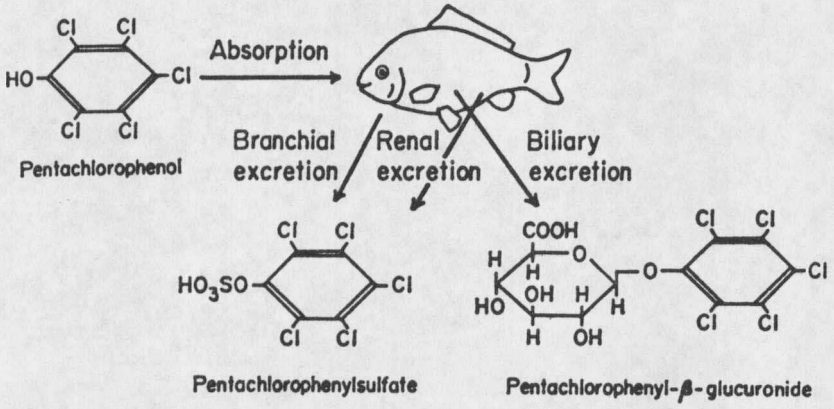


Figure 1. The major detoxification pathways for PCP in fishes. (From Kobayashi and Nakamura, 1979).

# CHAPTER 2

## METHODS

Two flow through toxicity tests were conducted on cutthroat trout to determine possible pathologic effects in the liver or gills induced by PCP. The tests were conducted at the MSU/FBL. The PCP used in this study was obtained from a stock supply at the MSU/FBL. It was reported to be comparable to that of a 98% PCP, Lot #6-192 obtained from Chem Service, West Chester, PA 19380.

The water source for the tests was a natural groundwater spring located at the U.S. Fish and Wildlife Service, Fish Cultural Development Center (FCDC), Bozeman, Montana. Chemical characteristics of this spring water are shown in Table 1 and a summary of water chemistry analyses are presented in Table 2.

Temperature, dissolved oxygen, and pH were determined for water in the test tanks as follows: dissolved oxygen was measured using a Yellow Springs Instrument (YSI) Model 54 A Oxygen meter, temperature with a calibrated mercury thermometer, and pH with a Beckman Model 3500 Digital pH meter. Total alkalinity, total hardness, and specific conductance were determined in accordance with the American Public Health Association (APHA), Standard Methods for the Examination of Water and Wastewater (3). Chemical analyses from a previous PCP study, 1 week prior to this study, were used to report total alkalinity, total hardness, and specific conductance. These three variables

Table 1. Chemical characteristics of the dilution water.

| | |
|---|---|
| Al < 0.02 mg/l | Mg 9.8 mg/l |
| B < 10.0 mg/l | Mn 6.0 mg/l |
| Ba 31.0 µg/l | Mo < 0.05 mg/l |
| Be < 2.0 µg/l | Na 0.6 mg/l |
| Ca 45.9 mg/l | Ni < 0.05 mg/l |
| Cd < 0.5 mg/l | P < 1.0 mg/l |
| Cr < 0.1 mg/l | Pb < 0.3 mg/l |
| Cu < 2.0 µg/l | Si 0.3 mg/l |
| K 0.48 mg/l | Sr < 0.05 mg/l |

Table 2. Summary of water chemistry analysis
Test No. 2

| DATE | 4-16-82 | 4-19-82 | 4-19-82 | 4-9-82 | 4-9-82 | 4-8-82 |
|---|---|---|---|---|---|---|
| TANK | D.O. (mg/1) | pH | Temp. °C | Alk. (mg/1)* | Hardness* mg/1 | S.E.C.* µ mhos |
| 1 | 8.3 | 7.64 | 11.4 | 173 | 206 | 391 |
| 2 | 8.2 | 7.69 | 10.9 | 172 | 206 | 392 |
| 3 | 8.0 | 7.73 | 10.9 | 172 | 205 | 394 |
| 4 | 7.9 | 7.70 | 10.9 | 173 | 205 | 394 |
| 5 | 7.3 | 7.79 | 11.3 | 173 | 205 | 399 |
| 6 | 7.4 | 7.89 | 10.9 | -- | -- | -- |

*Measurements from a similar PCP bioassay

are relatively constant in the water supply used, and would be expected to be similar in both tests.

The PCP toxicant solution was made by placing 2 grams (g) of PCP in each of two 20-liter napthalene containers. These were then filled with 20 liters (1) of spring water and allowed to stand, with occasional agitation, for 24 hours prior to starting the bioassays. 2 g in 20 1 exceeds by 25% the aquatic solubility (80 mg/1) of PCP (41).

Stock toxicant was added to each of five test tanks (#2-#6) by means of a proportional diluter (dilution factor 0.75) of the basic design of Mount and Brungs (30); a sixth tank served as the control (#1). The water flow rate from the diluter into each tank was 500 ml every 2 to 3 minutes. Water replacement time was approximately 2 hours, and full test concentration was effectively reached within 8 hours.

Water was analyzed for PCP twice during the tests (on day 2 and day 4), using gas chromatography by the following method: extraction of PCP from water samples was accomplished at room temperature by acidifying a 125 milliliter (ml) water sample to a pH of less than 2 with 200 microliters (ml) of concentrated $H_2SO_4$. Fifty ml of this acidified sample were then placed in a 100 ml volumetric flask and 50 ml of toluene were added. This mixture was stirred using a magnetic stirring device for 1 hour and then allowed to settle for 1 hour. A 1 ml aliquot of sample extract was then derivitized using a solution of 2 ml ether, 2 ml hexane, and 100 ml of diazomethane. The sample was allowed to equilibrate for 1 hour and then analyzed using

gas chromatograph electron capture detection (2). Specific gas chromatograph settings are listed in Table 3.

Prior to testing, fish were reared at 10 C under hatchery conditions at the FCDC.

Fish were exposed to toxicant in glass tanks (50 x 25 x 30 cm) containing a test water volume of 21.8 1. Figures 2 and 3 show the operational system employed in this study. Fish were acclimated to the test tanks for 24 hours prior to introduction of toxicant and were not fed during the acclimatization period nor during the test.

This study differed from a typical 96-hour acute toxicity test in that its primary objective was to obtain numerous fish samples for histologic examination. To accomplish this the maximum carrying capacity for each tank needed to be determined. The "Flow Index" formula (34) used by fish culturists to determine carrying capacity of fish rearing units was used to determine the permissible number of fish that could be held in each test tank, without inducing unwanted metabolites, which could influence the toxicity test results (Table 4).

From a rearing tank, holding approximately 5,000 cutthroat trout, 40 fish were randomly sampled and individually weighed and measured. Average values were used to determine the permissable number of fish to be placed in each test tank. A total of 75 fish were placed in each tank (Table 5).

During the toxicity tests, observations were made at 4-hour intervals. Moribund fish were removed at the observation times and prepared for histologic examination. Dead fish, as evidenced by

Table 3. Gas chromatograph settings for PCP standard curve

---

Compound: PCP

Concentration Range: 0.02 - 0.5 mg/l

Column: 2 ft. x 1/4" o.d. x 2 mm i.d. glass column packed with 1.5% OV-17, 1.95% QF-1 on 80/100 mesh (Applied Science, State College, PA)

Oven Profile: Gas Chrom Q

Oven Temperature: 140°C

Injector Temperature: Isothermal

Detector: Electron capture

Detector Temperature: 300°C

Attenuation:

Sampling Method: Hewlett Packard 7672A Autosampler, 1 µl injections
Hewlett Packard 5880A Gas Chromatograph
(Hewlett Packard, Avondale, PA)

Comments:

Standards were 0.025, 0.05, 0.1, 0.5 mg/l. 50 ml samples were extracted for 1 hour with 50 ml toluene using 100 ml volumetric flasks and a magnetic stirring apparatus.

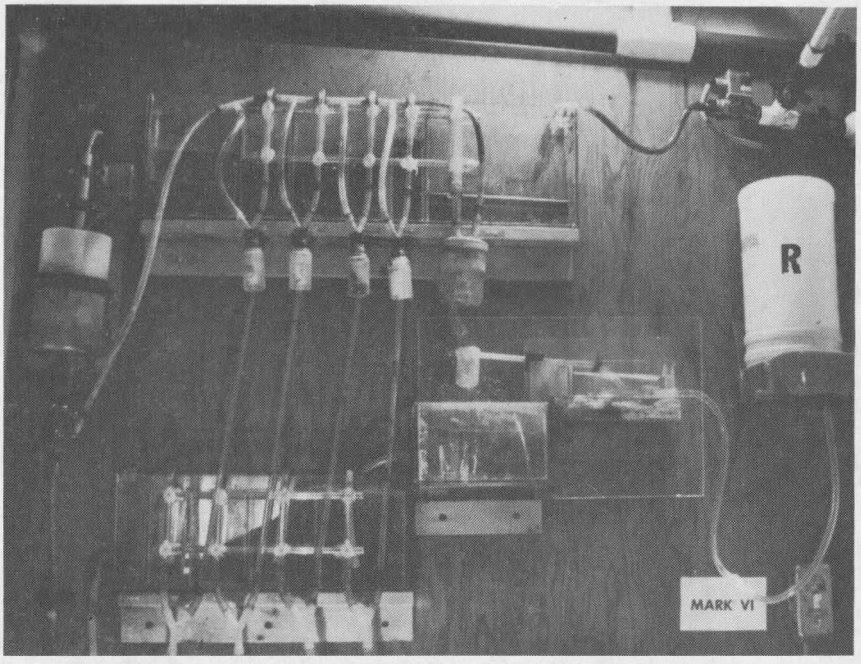


Figure 2. Proportional diluter. (R) toxicant reservoir.

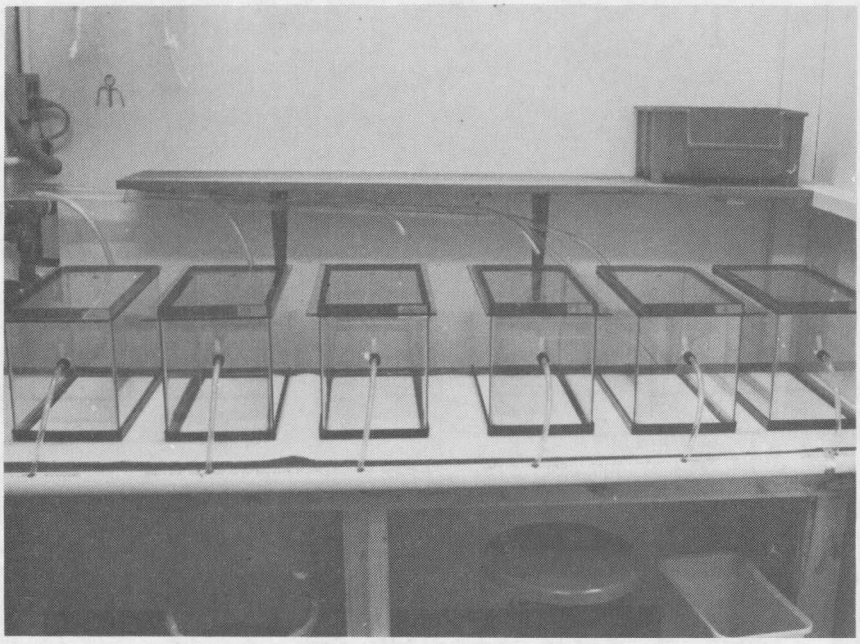

Figure 3. Test tanks.

Table 4. Flow Index formula

| |
|---|
| W = F L I |
| W = total fish weight (pounds) |
| F = loading factor (1.5) |
| L = fish length (inches) |
| I = water inflow (gallons per minute) |

Table 5. Carrying capacity of test tanks

Flow into test tanks = 12 liters/hour (0.053 gpm)

Fish length = 1.85 inches

Fish weight = 0.89 grams/fish

Loading factor (F) = 1.5 (established empirically for this water supply)

$W = F\ L\ I = (1.5)\ (1.85)\ (0.053) = 0.147$ pounds

0.147 pounds = 66.74 grams

$$\frac{66.74 \text{ grams}}{0.89 \text{ grams/fish}} = 75 \text{ fish per tank}$$

complete immobilization and lack of operculation were not used for histologic examination because of possible post-mortem autolysis (29).

Fish taken for light microscopic examination were incised ventrally to maximize exposure of internal organs, and fixed in Bouin's solution for 12 to 24 hours. Samples were then transferred to 65% ethanol until processing. Whole fish were embedded in paraffin, sectioned at 5 microns (m) and stained with hematoxylin and eosin. Gill and liver tissues were examined histologically in an attempt to identify any cellular alterations induced by PCP. These two organs were specifically chosen because the gill is considered a major absorptive, and excretory organ and the liver is a major detoxifying center for xenobiotics.

Fish collected for electron microscopy were placed directly into a solution of 4% formaldehyde and 1% glutaraldehyde, buffered to a pH of 7.2 in phosphate buffer (45). After 5 to 10 minutes, the left operculum was removed from each fish to expose the gills, the fish was incised ventrally, and visceral organs were pulled from the body cavity to maximize exposure to fixative. After 2 to 3 days in fixative, a number of filaments from the outermost left gill arch and a small portion of liver from each sample fish were post-fixed in 1% aqueous osmium tetroxide, dehydrated in graded ethanol, and embedded in Spurr's embedding medium (41). Silver-gold (50 to 70 nanometers) sections were placed on copper grids and stained with uranyl acetate and Reynold's lead citrate (27). They were examined at 80 to 100 Kv with a JEOL - 100 CX transmission electron microscope.

Liver and gill tissues from the same fish collected for electron microscopy were also processed for light microscopy. These samples were embedded in glycol methacrylate, sectioned at 3 m and stained with hematoxylin and eosin.

In addition to collecting moribund fish for examination, fish were also collected at the termination of the 96-hour test. At this time, 10 fish for light microscopy (LM) and 5 for electron microscopy (EM) were preserved from each of tanks 1 thru 4. At termination only 13 fish remained in tank 5; therefore, only 4 fish were collected for each LM and EM. All fish in tank 6 were dead by the end of 96 hours.

# CHAPTER 3

## RESULTS

A decrease in dissolved oxygen as toxicant concentration increased was observed. This is probably due to the increased respiration of test fish induced by exposure to PCP. However, even the lowest reported dissolved oxygen concentration (7.3 mg/l) is more than adequate for the cutthroat trout, under the experimental conditions of this study (35).

The concentrations of PCP to which fish were exposed are presented in Table 6. Test #1 exposed fish to acutely lethal concentrations of PCP in all test tanks. At the highest exposure concentration (tank #6, 0.630 mg/l PCP) death occurred rapidly, with all fish succumbing after only 2½ hours exposure. At the lowest exposure concentration (tank #2, 0.192 mg/l PCP) all fish had succumbed after 8 hours. In test #2, fish were exposed to both acutely lethal and sublethal concentrations of PCP, with the highest exposure concentration (tank #6) averaging 0.165 mg/l (range 0.125 - 0.200) PCP. Mortality at this concentration began to occur after 20 hours (Figure 4). After 40 hours, 19 of the original 75 fish remained alive, with no more mortality occurring for the next 28 hours (68 hours total). After 64 hours had elapsed in the test more PCP stock solution was added to the reservoir of the proportional diluter. The stock PCP had apparently become more saturated upon standing because mortality

Table 6. PCP water analyses (mg/1)

| Test No. 1 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Date | Time | Tank 1 | Tank 2 | Tank 3 | Tank 4 | Tank 5 | Tank 6 |
| | | | | * | | * | |
| 4-11 | 0900 | 0.000 | 0.192 | 0.272 | 0.362 | 0.473 | 0.630 |

* calculated using dilution factor 0.75

| Test No. 2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Date | Time | Tank 1 | Tank 2 | Tank 3 | Tank 4 | Tank 5 | Tank 6 |
| 4-16 | 0900 | 0.000 | 0.040 | 0.050 | 0.069 | 0.084 | 0.125 |
| 4-19 | 0900 | 0.000 | 0.058 | 0.077 | 0.102 | 0.128 | 0.200 |
| | Mean | 0.000 | 0.049 | 0.064 | 0.086 | 0.106 | 0.165 |

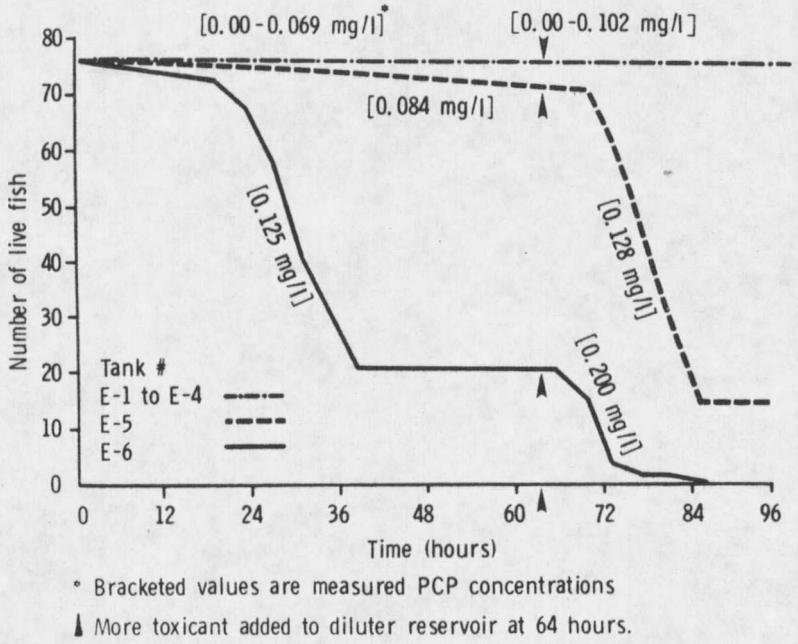


Figure 4. Mortality versus exposure time and PCP concentration.

began occurring once again at the highest exposure concentration (tank #6) and also began occurring at the second highest concentration (tank #5) (Figure 4).

Although these tests weren't intended to establish an LC 50 value for PCP to cutthroat trout, the value would be between 0.084 mg/l and 0.125 mg/l according to these results.

## Clinical Signs

Clinical signs in cutthroat trout exposed to lethal concentrations of PCP included: 1) increased and labored operculation (respiration), 2) lethargy and swimming at water surface, 3) loss of equilibrium (twirling), 4) inability to maintain position in the water column (laying on the tank bottom) and 5) death.

Clinical signs in fish exposed to sublethal concentrations of PCP were limited to increased operculation (respiration).

## Normal Histology

Gills: The trout gill consists of gill arches, gill filaments and gill lamellae. There are 4 gill arches under each operculum. Two rows of gill filaments are present on each arch and the lamellae branch from the filaments. The gill lamellae are the primary sites of respiration and also function in excretion, absorption, and osmoregulation. They are supplied with blood from the afferent filament artery via the afferent lamellar arterioles. Blood leaves the lamellae via efferent lamellar arterioles. The lamellae appear as leaflike projections that arise alternately from the upper and lower

surfaces of the gill filaments. The epithelium of the gill lamellae consists of a layer of overlapping and/or interdigitating squamous epithelium that is supported by a basement membrane and a thin layer of connective tissue. Contractile pillar cells support the lamellae and have flanges which surround the blood sinusoids. The lamellae are open, except for the pillar cells which separate one side of a lamellae from the other. Chloride cells, the primary osmoregulatory cell in fishes, are generally located at the base of and between the gill lamellae (28) (Figure 5).

Liver: Liver parenchyma in cutthroat trout is not divided into distinct lobules. It is composed of branching and anastomosing, two cell thick laminae of hepatocytes that are separated by distinct endothelial lined sinusoids. In cross section these may appear as rosettes of several cells radially arranged about sinusoids (Figures 6 and 7). Each hepatocyte has an irregular polygonal shape and most contain a single spherical nucleus with a distinct nucleolus. A normal complement of organelles, including mitochondria, endoplasmic reticulum, lysosomes, and golgi are easily recognizable (10) (Figures 8 and 9).

Hatchery reared salmonids typically have large amounts of glycogen within the hepatocytes that appear histologically as extensive

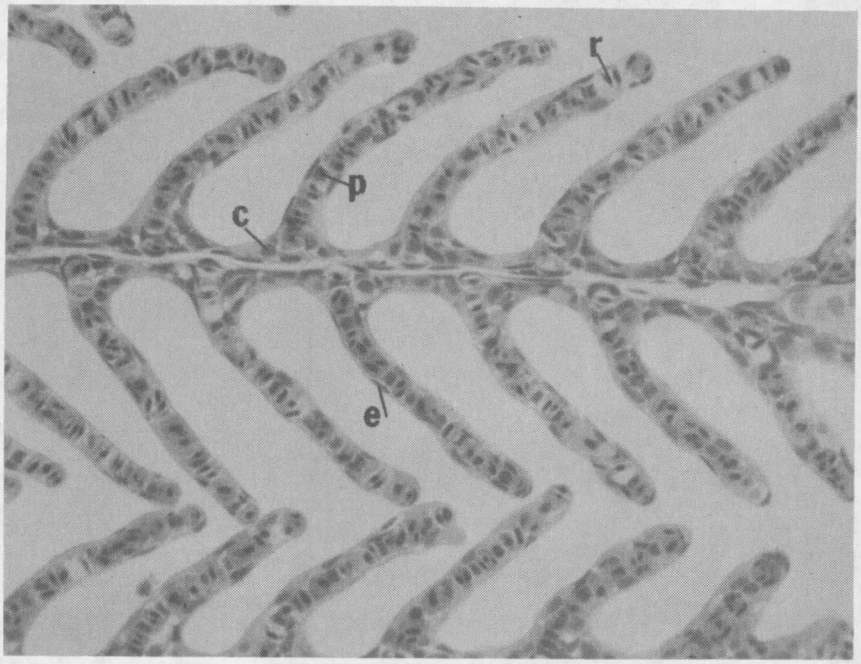


Figure 5. Light Micrograph (LM). Control gill from cutthroat trout. Gill lamellae projecting from gill filaments. (c) chloride cell, (e) squamous epithelium, (p) pillar cell, (r) nucleated red blood cell (rbc) in blood sinusoid. x 175, H & E.

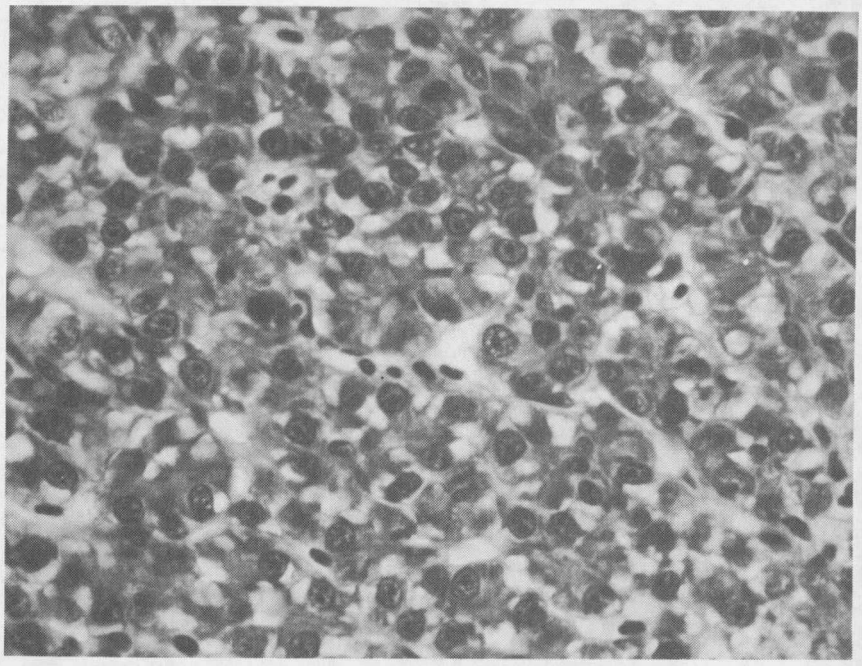

Figure 6. LM. Control (normal) liver. Cutthroat trout. Note: glycogen vacuolation and anastomosing laminae of hepatocytes. x 475. H & E.

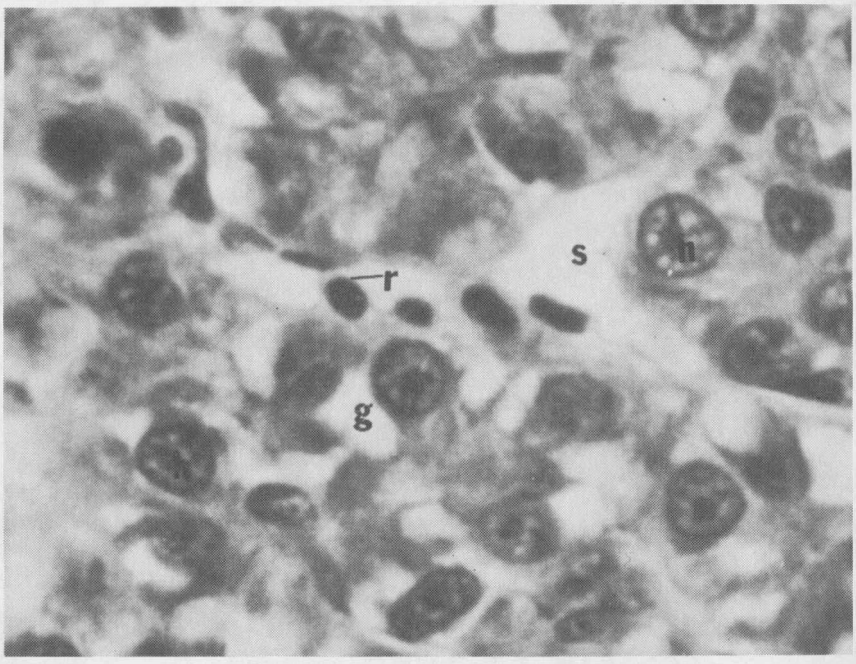


Figure 7. LM. Control (normal) liver. Note: glycogen vacuolation (g), hepatocyte nuclei (h), red blood cell (r) in sinusoid (s). x 1190, H & E.

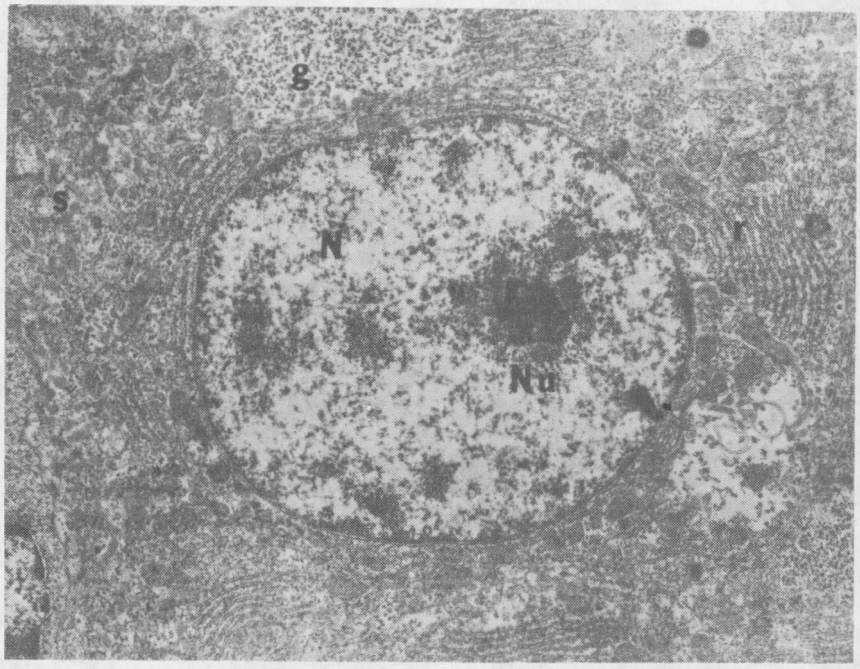


Figure 8. Transmission Electron Micrograph (TEM). Hepatocyte from a control cutthroat trout. (N) nucleus, (Nu) nucleolus, (g) glycogen particles, (r) granular endoplasmic reticulum (GER), (s) agranular endoplasmic reticulum. Many mitochondria are also evident. x 7,700.