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CHANGES IN HYDROMINERAL BALANCE, STRESS AND GILL HISTOLOGY OF RAINBOW TROUT *(Salmo gairdneri)* CHRONICALLY EXPOSED TO COPPER

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled CHANGES IN HYDROMINERAL BALANCE, STRESS AND GILL HISTOLOGY OF RAINBOW TROUT *(Salmo gairdneri)* CHRONICALLY EXPOSED TO COPPER submitted by JUNE RITA WILLIAMS in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The ability of rainbow trout (*Salmo gairdneri*) to adapt during chronic (up to 64 days) exposure to copper (20-120 µg/L Cu) was investigated. The following parameters, related to ionic and osmotic regulation and the stress response, were measured: total body water and the plasma concentrations of Cl^-, Na^+, K^+ and glucose. Changes in the gill structure of fish exposed to 0 and 120 µg/L Cu were monitored concurrently, using quantitative morphometric techniques adapted to light microscopy, to determine if any changes in gill structure would be correlated with hydromineral changes or the stress response.

Transient changes occurred in the plasma concentrations of Cl^-, Na^+ and glucose of trout following exposure to 120 µg/L Cu and in the plasma Cl^- concentration of trout following exposure to 50 and 80 µg/L Cu, demonstrating the ability of the trout to adapt to copper. However, recovery in the levels of the plasma parameters did not occur by the end of the experiment in some of the trout exposed to 120 µg/L Cu, indicating variation in the ability of trout to adapt to copper.

The plasma K^+ levels of trout exposed to 100 and 120 µg/L Cu declined over the duration of the experiment.

No significant changes in total body water occurred during exposure of the trout to copper; but, significant negative correlations were found between total body water and the plasma levels of both Cl^- and Na^+ of trout exposed to 120 µg/L Cu, suggesting that part of the reduction in plasma ion levels (before recovery) may have been due to dilution.

A strong association between the level of stress and the reduction in plasma ion levels due to copper exposure was suggested by the highly significant ($p = 0.001$) negative correlations between plasma glucose and the plasma levels of both Cl^- and Na^+.

A reduction in the proportion of gill epithelia may be associated with reductions in the plasma levels of Cl^- and Na^+ and with an increase in plasma glucose levels. An increase in the relative volume of the blood channel spaces in the gill pillar system was associated with a reduction in the plasma K^+ levels.

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INTRODUCTION

Copper, a common aquatic pollutant in mining areas (Cook and Hoos, 1971; Van Diepen, 1975; Wells *et al.*, 1974) is one of the most toxic of the heavy metals to fish (Oshima, 1931; Pickering and Henderson, 1966). In spite of the high toxicity, copper-polluted waters have been found to support endemic fish populations but to kill hatchery-reared fish of the same species (Paul, 1952), indicating that adaptation to copper may be possible.

The ability of fish to adapt to copper has been indicated by several laboratory studies (Christensen *et al.*, 1972; Dixon, 1978; Lewis and Lewis, 1971; McKim *et al.*, 1970; Miller, 1980; Waiwood and Beamish, 1978). Dixon (1978) and Miller (1980) demonstrated that the LC_{50} (lethal concentration killing 50% of the fish) of copper to rainbow trout (*Salmo gairdneri*) was higher in fish pre-exposed to sublethal concentrations of copper than in fish with no previous exposure to copper.

Adaptation to an environmental change can be demonstrated by a return of physiological parameters to pre-exposure levels or by attainment of a new steady-state in the parameters (Wilson, 1972). Transient changes in plasma chloride, serum and plasma osmolality, plasma glucose, hematocrit and swimming performance have been noted in fish exposed to sublethal concentrations of

copper (Christensen *et al.*, 1972; Lewis and Lewis, 1971; McKim *et al.*, 1970; Waiwood and Beamish, 1978). Many other studies have noted changes in various physiological and behavioral parameters of fish exposed to copper, but the studies were of insufficient duration to determine if adaptation was evident (review by Spear and Pierce, 1979).

The first objective of the present study was to examine the time course of changes in some physiological parameters of fish (*Salmo gairdneri*) chronically exposed to sublethal concentrations of copper. Parameters related to ionic and osmotic regulation were chosen because: (i) copper has been shown to influence the levels of ions and water in fish (review by Spear and Pierce, 1979), (ii) previous chronic exposure studies that measured ion changes (Christensen *et al.*, 1972; McKim *et al.*, 1970) had too few sampling periods to determine the time course of the changes, and (iii) previous chronic exposure studies did not simultaneously measure both ionic concentration and total body water changes in the fish. The parameters measured in the present study were: the plasma concentrations of chloride, sodium and potassium, and the quantity of water in the whole fish.

Since the hydromineral balance in fish may also be influenced by the endocrine system (Wilson, 1972) and ce stress of many kinds influences the blood levels

of hormones in fish (review by Mazeaud *et al.*, 1977), an index was obtained of the stress levels experienced by fish used in the present study. Plasma glucose concentration has been used as a measure of stress in previous studies (Silbergeld, 1974; Mazeaud *et al.*, 1977) and was also used for that purpose in the present study.

The fish gill is an important semi-permeable membrane involved in ionic and osmotic regulation (Maetz, 1974), so morphological changes in the gill would be expected to influence ion and water balance. Qualitative histological studies have revealed that structural changes in gills of fish exposed to lethal levels of copper included detachment of the epithelia from the basement membrane (Bilinski and Jonas, 1973; McFadden, 1965) and fusion of the secondary lamellae (Baker, 1969). On exposure to sublethal levels of copper, fish gills have shown vacuolation (Baker, 1969), a decrease in the number of mucous cells (Baker, 1969; Labat *et al.*, 1974) and a reduction in the thickness of the epithelium in the basilamellar region (Baker, 1969).

Relatively few studies, though, have made quantitative measurements on fish gill structure (review by Hughes, 1972) and even fewer studies have quantified changes due to pollutants (Hughes and Perry, 1976; Hughes *et al.*, 1979). In order to evaluate statistically possible relationships between gill structure and the level of ions in the plasma or the body water content of the fish,

quantification of the structural changes is necessary. To date no study has been reported that simultaneously quantified structural changes in fish gills and physiological changes in fish exposed to a pollutant. Furthermore, no study has quantified the structural changes in the gills of fish chronically exposed to copper.

Therefore, the final objective of the present study was to quantify changes in the gill structure of trout chronically exposed to copper and to relate these changes to any changes in the physiological parameters measured simultaneously. Since a change in the amount of epithelium or in the surface area of the gill may influence the movement of ions and water between the external environment and the blood of the fish, the epithelial volume and the surface area of the gill lamellae were estimated. The relative volume of the blood channel spaces in the secondary lamellae of the gills was also estimated because changes in the size of the blood channel spaces had been noted in fish exposed to zinc (Skidmore and Tovell, 1972) and to ammonia (Smart, 1976). Whether changes in the blood channel spaces would be correlated with hydromineral changes in fish is not known.

In summary, the present study was conducted to determine whether:

(i) the hydromineral balance in trout would show adaptation to chronic copper exposure,

(ii) the level of stress experienced by trout during chronic copper exposure would show adaptation and, if so, whether the level of stress was correlated with any hydromineral changes,

(iii) structural changes occurred in trout gills during chronic exposure to copper and, if so, whether the structural changes were correlated with any hydromineral changes.

MATERIALS AND METHODS

I. Experimental Animals

Source and holding conditions

Sexually immature rainbow trout, *Salmo gairdneri*, (33.5 ± 11.6 g; mean ± S.D) were obtained from Trout Springs (Tacoma, Washington, U.S.A.) and from Allen's Trout Farm (Calgary, Alberta). The fish from Allen's Trout Farm had been imported as fingerlings from Trout Springs.

Most trout were held at least two weeks in tanks continuously supplied with City of Edmonton water which had been treated by: circulation through charcoal filters, addition of sodium thiosulphate to inactivate all residual chlorine, and addition of sulphuric acid to maintain a pH of 7.8. The water temperature was maintained at 5 ± 1°C and the photoperiod was adjusted to follow the natural regime. All fish were fed trout food pellets (Ewos, Astra Chemicals Ltd., Ontario) every second day.

Acclimation

The experimental fish were transferred to an acclimation tank and held there for a minimum of nine days before use in an experiment. The tank held approximately 240 L of synthetic freshwater (described

below) and had a continuous inflow of newly synthesized freshwater.

The photoperiod (10L:14D) and temperature (10 ± 1°C) were constant for all experiments. Water temperature was maintained by two methods: (i) immersion of plastic-coated cooling coils into the storage tank from which all the synthetic water was siphoned and (ii) maintenance of the room temperature at 10°C. The water temperature occasionally rose above 10°C (Appendix I) during the hot summer months when the room temperature could not be maintained at 10°C.

Approximately 75% of the experimental fish were treated for fin rot during the acclimation period (Tables 1 and 2). The fish were anesthetized in tricaine methane sulphonate (Kent Laboratories or Sigma Chemical Co.) and the diseased fin tissue was cut off. The cut areas were then painted with a water-soluble iodine solution (Betadine Solution, Purdue Frederick Company, Toronto). Regeneration of healthy fin tissue was apparent one to two weeks after treatment.

The fish were fed with Ewos trout food pellets every second day throughout the acclimation and test periods. Debris was siphoned out of the tanks as required, usually once daily.

II. General Test Conditions

Test tanks

Fiberglas tanks which held approximately 115 L of water were used for the experiments. Each tank was supplied with a Fiberglas screen cover and a polyvinyl chloride overflow standpipe. The screen covered two-thirds of each tank and the remaining third of each tank was covered by a removable green Fiberglas sheet to permit access to the fish.

Prior to any experiment, all tanks were scrubbed and disinfected with bleach or with Betadine Solution. Distilled water, acidified with nitric acid, was then left in each tank overnight to leach out any copper that may have adsorbed onto the walls of the tank. The tanks were finally rinsed and filled with the synthetic freshwater.

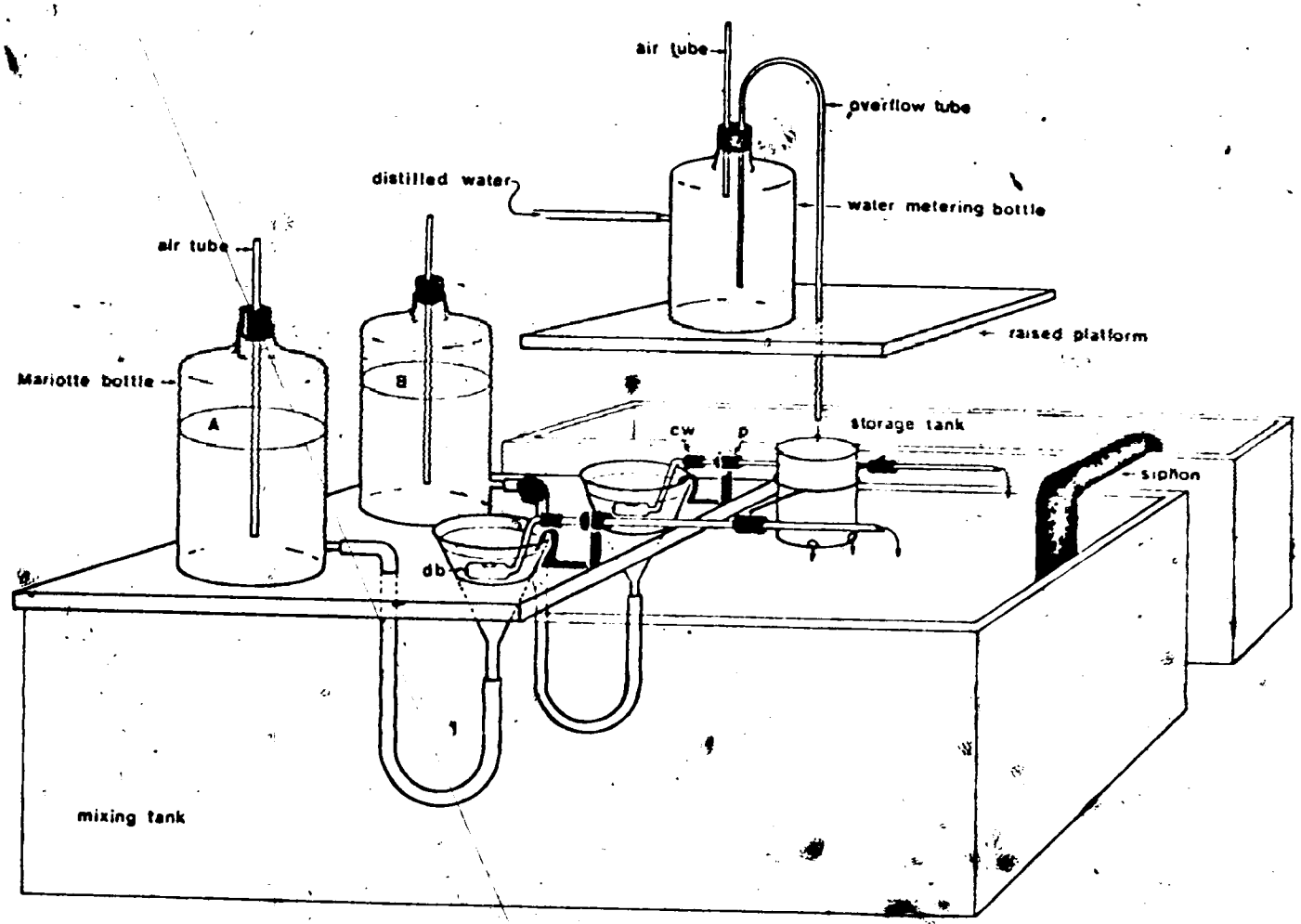
Water supply

A synthetic freshwater was used for the experiments for two reasons: (i) the sodium thiosulphate in the treated city water could have bound the copper added to the test tanks, decreasing the toxicity of the solution (Nishikawa and Tabata, 1969) and (ii) toxic substances, including significant levels of copper (approximately 10 µg/L Cu), were often present in the city water.

The synthetic freshwater was made from concentrated stock solutions of salt and distilled deionized water (distilled water which had been passed through a resin ion exchange filter, Barnstead - standard high capacity). The final concentrations of the salts in the synthetic freshwater and the quality of the salts used were: 1.2 mM calcium chloride ($CaCl_2 \cdot 2H_2O$, U.S.P. grade), 0.4 mM sodium bicarbonate ($NaHCO_3$, U.S.P. grade) and 0.04 mM potassium bicarbarbonate ($KHCO_3$, reagent grade). The water quality analysis is listed in Appendix I.

A dosing apparatus, modified from Mount and Brungs (1967), was used to make the synthetic freshwater (Figure 1). A constant volume of the distilled water was discharged at regular intervals from a water metering bottle. The amount of water discharged and the time interval between each discharge was dependent on the rate at which the water entered the water metering bottle. The bottom of the glass air tube in each Mariotte bottle determined the level of stock salt solution in the attached funnel. Thus, the amount of solution filling each dipping bird was held constant. Whenever the water was discharged from the water metering bottle into the bucket attached to the arms of the dipping birds, the added weight caused the dipping birds to rotate on their pivots and to empty a calibrated amount of the salt solutions into the mixing tank. When the water drained out of the bucket,

Figure 1. Dosing apparatus used to make synthetic freshwater - modified from Mount and Brungs (1967). Mariotte bottle A contained the $CaCl_2 \cdot 2H_2O$ stock solution. Mariotte bottle B contained the $NaHCO_3$-$KHCO_3$ stock solution. db-dipping bird; cw-counterweight; p-pivot.


air tube
overflow tube
distilled water
water metering bottle
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Mariotte bottle
A
B
cw
p
storage tank
siphon
db
mixing tank

the counterweights caused the dipping birds to drop back into the funnels for refilling. Rapid mixing of the distilled water and the salt solutions was ensured by vigorous aeration of the water in the mixing tank below the entry point of the salt solutions. Since the flow rate of the distilled water entering the water metering bottle varied slightly due to pressure variations in the water line, the amount of water discharged at one time was measured periodically so that the concentration of the stock solutions could be adjusted to maintain the composition of the synthetic water.

The synthesized freshwater from the mixing tank was then siphoned to the storage tank and cooled by the cooling coils. From the storage tank, the water was siphoned to the individual test and acclimation tanks. Each siphon from the storage tank consisted of polyethylene tubing fitted with plexiglass plugs. A narrow hole had been drilled into each plug to reduce water flow to approximately 250-300 mL/min.

Addition of toxicant

Copper was added to each test tank by means of an individual dosing apparatus similar to the one used to make the synthetic freshwater. Each Mariotte bottle was filled with a concentrated solution of copper sulphate ($CuSO_4 \cdot 5H_2O$, reagent grade) made with distilled deionized

water and acidified with 5 mL/L concentrated nitric acid (reagent grade). The concentrations of the stock copper sulphate solutions were adjusted periodically to yield the desired nominal concentrations of copper in the test tanks, calculated as total copper. The stock solution for the control tank consisted of distilled deionized water acidified with 5 mL/L concentrated nitric acid.

III. Experimental Procedures

A. Determination of the Median Lethal Concentration (LC_{50}) of Copper.

A series of three 10-day tests were conducted to determine the concentration of copper that caused 50% of the trout to die (LC_{50}).

The experimental protocol for these tests is outlined in Table 1. For tests B and C, the fish were placed into the test tanks prior to the addition of copper so that the fish would have time to recover from the stress of handling (Wedemeyer, 1972) before being subjected to a copper-induced stress. The measured copper concentrations, water quality parameters and water turnover rates for these tests are listed in Appendix IA.

In each test, the tanks were checked for dead fish every four hours except for 0400 MDT (Mountain Daylight Time). When warranted, the tanks were checked every two

TABLE 1. Experimental protocol for LC_{50} determinations

Test	Nominal Total Cu Concentration (µg/L)	Duration (Days) and Dates (1976)	No. Fish per Tank	Days Fish Acclimated To Experimental Conditions	Days Fish In Test Tank Prior To Cu Addition	Days Fin Rot Treatment Preceded Test
A	0,70,100,130	8 Jan 7-15	12	9	0	-
B	0,75,85,95	10 Feb 25-Mar 7	10	25	4	-
C	0,120,140,160	10 Sept 20-30	12	46	1	26

hours. A fish was considered dead if it was found ventral side up and if it did not move when prodded. The time of mortality of each fish was recorded. The weight, standard length and sex of each fish were also noted (Appendix II).

Test A was terminated after eight days of exposure due to leaking of the stock copper solutions into the 70 and the 100 µg/L Cu test tanks. During test B, the compressed air supply had been turned off without warning for four hours on day 2. Standby pumps were used within 1.5 hours of the shutdown but aeration may have been inadequate in some of the test tanks during the shutdown period since some of the air tubing was too short to reach the bottom of the tanks.

The rate of increase in total copper was monitored in the 95 µg/L Cu test tank during test B to determine the time required to reach the desired copper concentration (Appendix III).

B. Chronic Exposure to Copper.

1. General Experimental Protocol

In this experiment, trout were exposed to copper for a period of 64 days. It was felt that 64 days would be sufficient to determine whether the fish could adapt to copper and to determine the time course of the adaptation should it have occurred. The tests were conducted

in two series. The copper concentrations (calculated as total copper) used in series A were 0, 20, 50 and 80 µg/L Cu while the concentrations used in series B were 0, 50, 80, 100 and 120 µg/L Cu. The copper concentrations used in series A had been chosen on the basis of an LC_{50} of 80 µg/L Cu (Appendix VIIIA). However, during the chronic exposure experiment only three fish out of 44 had died in the 80 µg/L Cu exposure (Appendix X - Figure X-5), indicating that the LC_{50} was actually higher than 80 µg/L Cu. Therefore, the range of copper concentrations was increased for series B. The 8-day LC_{50} was later determined to be 103 µg/L Cu (see Results).

The experimental protocol for the chronic exposure tests is outlined in Table 2. All fish were acclimated to experimental conditions at least 18 days before use and were placed into the test tanks the day before the addition of copper was started. In the original design of the experiment all fish were to have been acclimated 18 days before use. However, due to higher than normal mortality of fish from toxic substances periodically entering the city water, many of the fish were transferred to acclimation conditions well before originally scheduled. The 100 µg/L Cu exposure was terminated after 16 days due to failure of the dosing apparatus.

Five fish were sampled on each of the following days of exposure: 0, 1, 2, 4, 8, 16, 32, 48 and 64.

TABLE 2. Experimental protocol for the chronic exposure tests

Series	Nominal Total Cu Concentration (µg/L)	Duration (Days) and Dates (1976)	No. Fish per Tank	Days Fish Acclimated To Experimental Conditions	Days Fish In Test Tank Prior To Cu Addition	Days Fin Rot Treatment Preceded Test
A	0	64 May 1-July 4	43	18	1	15
	20	64 Mar 24-May 27	46	33	1	-
	50	64 Mar 19-May 22	46	28	1	-
	80	64 Apr 10-June 13	44	18	1	15
B	0	64 July 7-Sept 9	50	60	1	54-57
	50	64 May 30-July 30	52	22	1	13-16
	80	64 June 24-Aug 27	51	47	1	41-44
	100	16 June 19-July 5	50	42	1	36-39
	120	48 July 24-Sept 10	49	19	1	11

In some cases fewer fish were sampled due to mortality during the experiment. Occasionally more than five fish were sampled if most of the fish survived or if a blood sample was noticeably hemolyzed. All fish were sampled between 0945-1115 MST (Mountain Standard Time) to minimize any differences due to diurnal changes in the parameters being measured. The starting times of each copper exposure were staggered (Table 2) so that no more than one group of fish was sampled on any given day.

2. Sampling Techniques

Water quality

Water samples to measure copper concentration were taken each time fish were sampled from a given tank. Two forms of copper were measured: total and dissolved. Samples for total copper were acidified with 2 mL/L concentrated nitric acid (reagent grade). Samples for dissolved copper were filtered through a 0.45 µm membrane filter (Gelman, GA-6) and then acidified with 2 mL/L concentrated nitric acid. All membrane filters were rinsed with dilute nitric acid before use. The acidified samples were then stored in acid-rinsed polypropylene bottles until analyzed. Due to some anomolous values for the total copper measurements additional water samples for total copper, taken during toxicity text C, were filtered through a 0.45 µm membrane filter after

acidification. A study on the effects of this treatment is reported in Appendix V.

Water samples from each test tank were collected periodically for measurement of the following water quality parameters: conductance, acidity, total alkalinity, calcium hardness, total hardness, chloride, sodium and potassium. The temperature, pH and dissolved oxygen content of each tank were also monitored. The methods used to measure these parameters are listed in Appendix VI. The measured copper concentrations, water quality parameters and water turnover rates are listed in Appendix IB.

Blood parameters

Blood was taken from unanesthetized fish by means of caudal puncture using a 19 gauge needle on a syringe which contained a small amount of 10% lithium heparin or ammonium heparin (Sigma Chemical Co.). In series A, the syringes for most of the sampling days were rinsed with heparin immediately before use. In series B and for some of the sampling days in series A (20 µg/L Cu - day 64; 80 µg/L Cu - days 48 and 64; 0 µg/L Cu - days 32, 48 and 64) the syringes containing heparin were air-dried before use. It was later determined that use of the syringes with liquid heparin diluted the blood sample between 1 and 5%. The dilution factor was not uniform across the samples since varying amounts of blood had

been collected.

Each blood sample was centrifuged (Beckman, Spinco Microfuge, model 152) twice, for one minute each time, to separate the plasma from the cells. The plasma was decanted after each centrifugation and frozen in polypropylene vials until analyzed.

Gill histology

Each fish was killed in an acutely lethal solution of tricaine methane sulphonate after the blood had been sampled. The entire gill was removed and immediately fixed in F.A.A. (formalin, acetic acid, alcohol - Lavdowski's Mixture; Corrington, 1941). The gills were kept in F.A.A. at least 48 hours then transferred to 70% ethyl alcohol for storage. The gills of fish used in series B were decalcified in RDO® (Dupage Kinetic Laboratories, Illinois) for five hours at some point during storage in ethyl alcohol.

Measurement of length and weight of fish

The wet weight (to the nearest 0.1 g) and the standard length (to the nearest 0.1 cm) of each fish were measured before the gills were excised. After removal of the gills, the wet and dry weights of each fish in series B were determined. For the dry weight measurements the fish were dried to a constant weight (within 0.005 g)

in an oven at temperatures between 65 and 85°C.

The fish measurements are listed in Appendix IV.

IV. Analytical Techniques

A. Water Quality

Copper was analyzed by an ammonium pyrrolidine dithiocarbamate (APDC)-methyl isobutyl ketone (MIBK) extraction procedure for atomic absorption spectrophotometry (Traversy, 1971). The ketone layer, which contained the copper extracted from a 100 mL sample, was aspirated into a Jarrell-Ash Flame Emission - Atomic Absorption Spectrophotometer (model 82-270). Absorption was measured at a wavelength of 324.7 nm. An acetylene:air mixture was used for the flame.

Standards were made fresh the day of analysis using double-distilled deionized water (distilled deionized water that had been redistilled) and a stock copper sulphate solution. All standards were acidified and filtered in the same manner as the water samples taken for copper analysis. The stock copper solution had been made with reagent grade copper sulphate ($CuSO_4 \cdot 5H_2O$) to a final concentration of 1000 mg/L copper and acidified with 2 mL/L concentrated nitric acid. The concentration of the standards versus the percent absorbance was plotted

on log-log paper. A straight line fitted through the standard points was used to determine the concentration of copper in the water samples taken from the test tanks.

The following water quality parameters were measured by G. Hutchinson of the Department of Zoology Water Laboratory: conductance, acidity, total alkalinity, calcium hardness, total hardness, chloride, sodium and potassium. The methods used to measure these parameters as well as pH, temperature and dissolved oxygen are summarized in Appendix VI.

B. Blood Parameters

Each plasma sample was thawed, mixed and recentrifuged before aliquots were taken for the analysis of Cl^-, Na^+, K^+ and glucose. Duplicate sub-samples were analyzed for each parameter. If the values of the duplicates were more than 3% apart, further sub-samples were analyzed until the values were within 3% of each other.

Plasma chloride concentrations were measured by titration with silver ions using a Buchler-Cotlove Chloridometer (Cotlove *et al.*, 1958). Ten µL of the plasma samples, blanks (double-distilled deionized water) and standard (60 mM/L NaCl, reagent grade) were each diluted in 4 mL of a nitric-acetic reagent (100 mL glacial acetic acid, 6.4 mL concentrated nitric acid and 900 mL double-distilled deionized water). Four drops of gelatin

reagent (Buchler Instruments Division, Nuclear-Chicago Corp.) were added before titration. The time of each titration was recorded and averaged for the replicates. The chloride concentration of each sample was then calculated as follows:

$$(\text{sample time} - \text{blank time}) \times \frac{\text{standard concentration}}{\text{standard time} - \text{blank time}}$$

The concentrations of sodium and potassium ions in the plasma samples were measured with a flame photometer using an internal lithium standard (Instrument Laboratories, Model 143). Four standard solutions, ranging from 0.0 Na^+/0.0 K^+ mM/L to 140 Na^+/5 K^+ mM/L, had been prepared from double-distilled deionized water and a commercial 140 Na^+/5 K^+ standard (Instrument Laboratories). Ten µL of the plasma samples and the prepared standards were each added to 2 mL of a 15 meq/L lithium solution (Instrument Laboratories). The plasma concentrations of Na^+ and K^+ were determined by comparing the readings obtained from the unknown samples to a standard curve plotted on arithmetic graph paper.

Plasma glucose concentrations were determined by an enzymatic colorimetric technique (Raabo and Terkildsen, 1960) using a Sigma Glucose Test Kit (Sigma Technical Bulletin No. 510, 1976). A Beckman/Spinco Spectro-Colorimeter (Model 151) was used to measure the absorbance of the samples and standards at a wavelength of 450 nm. The readings obtained from the unknown samples were compared

to a standard curve plotted on arithmetic graph paper to determine the plasma glucose concentrations.

C. Total Body Water

The percentage of water in the whole fish was determined on the fish used in series B as follows:

$$\% \text{ body water} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

D. Histological Techniques

Detailed histological examination, using a technique modified from Hughes and Perry (1976), was limited to the gills of the control fish and of the 120 µg/L Cu-exposed fish, series B. Three ratios were measured from the gill filaments: (i) the volume of the blood channel spaces relative to the volume of the pillar system (V_{BC}/V_{PS}), (ii) the volume of the epithelia in the gill lamellae relative to the volume of the entire secondary lamellae, including the epithelia of the primary lamella (V_{EP}/V_{SL}) and (iii) the surface area of the epithelial basement membrane attached to the pillar system (inner surface area) relative to the outer surface area of the epithelia (S_I/S_O). Ratios were determined to minimize systematic errors due to any differences in the plane of the sections (Hughes and Perry, 1976). The regions of the gill mentioned above are illustrated in Figure 2.

Figure 2. Example of the gill sections used for morphometric analyses.

A. Tracing of a typical field, illustrating the parameters measured.

EP epithelium

PC pillar cell

BC blood channel space

PS pillar system, equals PC + BC

SL secondary lamella

PL primary lamella

NT non-tissue space

SL' secondary lamella and adjacent epithelia of PL, equals EP + PS - NT

I epithelial basement membranes overlying PS (also referred to as inner surface)

O outer surface of epithelia

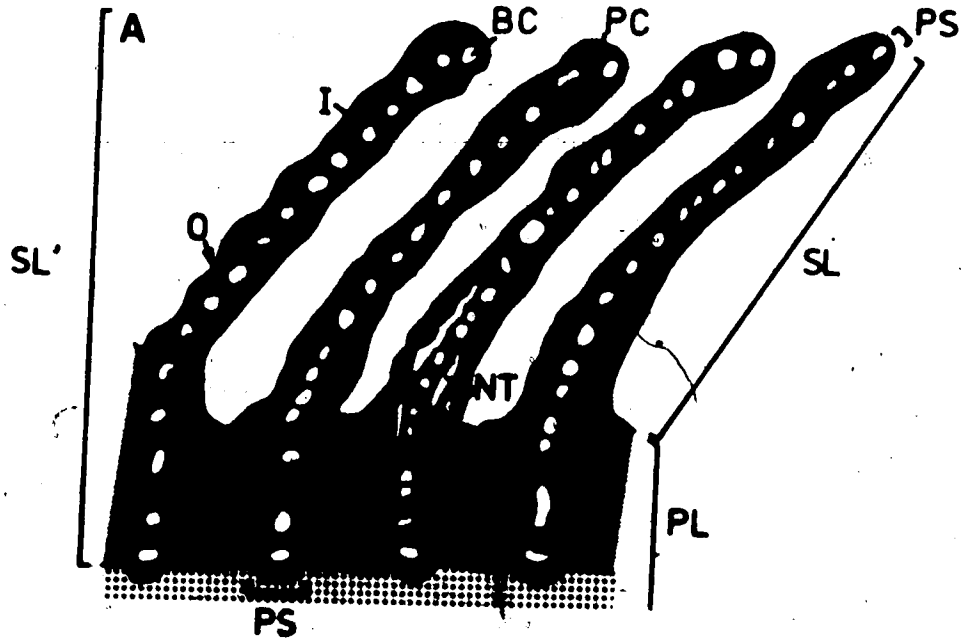
B. Photograph of a typical field with a Merz grid and a point grid superimposed. The grids are not to the scale actually used. Examples of some of the intersections and points counted are illustrated.

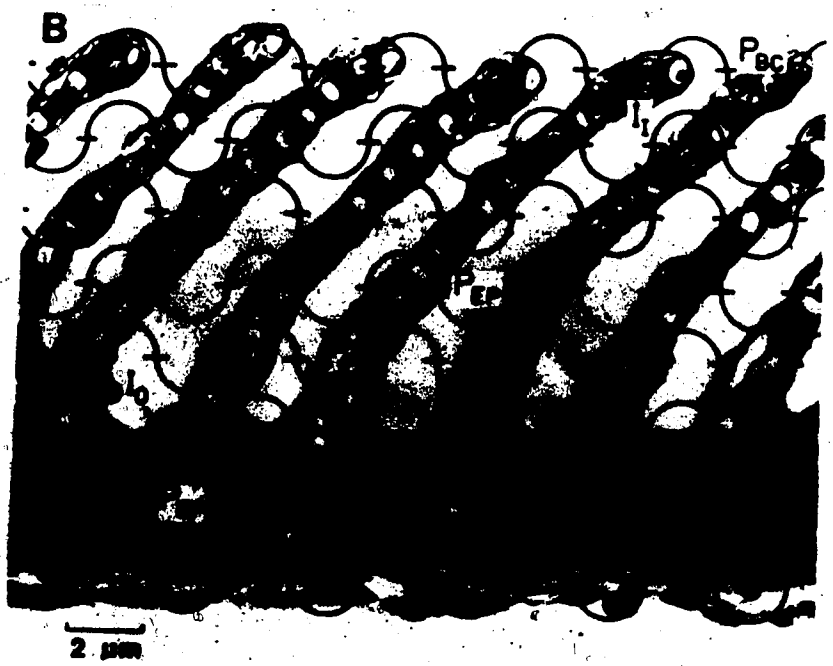
I_I intersection of Merz grid with inner surface

I_O intersection of Merz grid with outer surface of the epithelia

P_{EP} point within EP

P_{BC} point within BC


A
BC
PC
PS
I
O
SL'
SL
NT
PL
PS


B
P_{BC}
I_I
P_{EC}
I_O
2 μm

After fixation of the gills (see Materials and Methods IVD), the ventral portion of the second gill arch of each fish was embedded in paraffin (Paraplast) (Appendix VIIA) and sectioned to a thickness of 6-7 μm, perpendicular to the surfaces of the secondary lamellae. The sections were then mounted on clean glass slides and stained with Herovici's Polychrome (Herovici, 1963) (Appendix VIIB). Each slide was examined to locate gill filaments which had been cut longitudinally and perpendicular to the secondary lamellar surfaces for at least 4/5 of the filament length. Of the acceptable filaments, one filament per gill was selected, using a table of random numbers, for morphometric analysis.

Since structural changes may have differed along the length of the gill filament, each filament was divided into thirds (Figure 3). Photographs at two magnifications (200x and 400x) were taken from each third using black and white film (Panatomic-X, Eastman Kodak). The film negatives were projected onto the screen of a microfilm reader (Dea Graph, model RF6) yielding a final magnification of 1540x for the 200x photographs and of 3100x for the 400x photographs. Volumes were estimated by counting the number of points which were located over each tissue region using a regularly spaced point grid while surface areas were estimated by counting the number of intersections between the surfaces and the lines of a

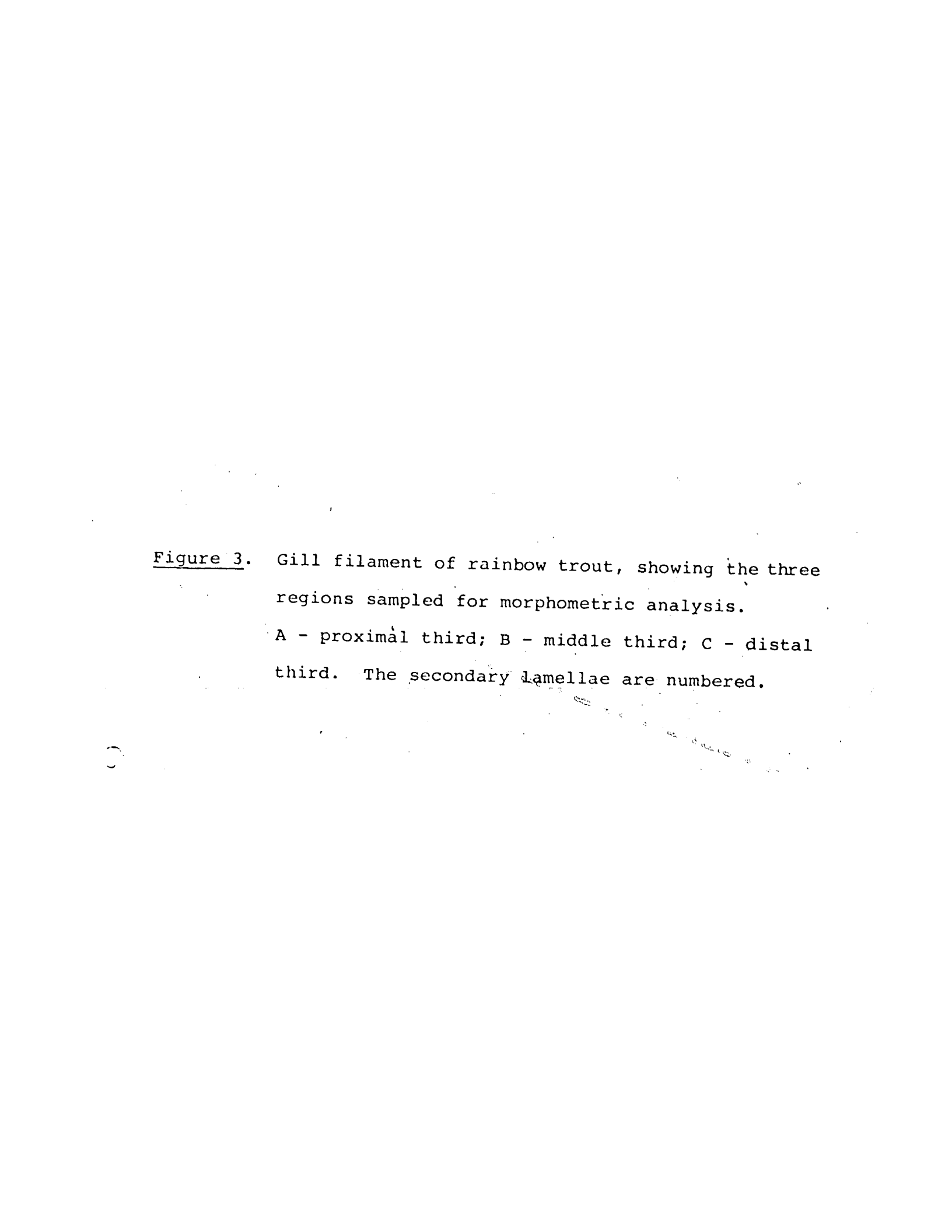
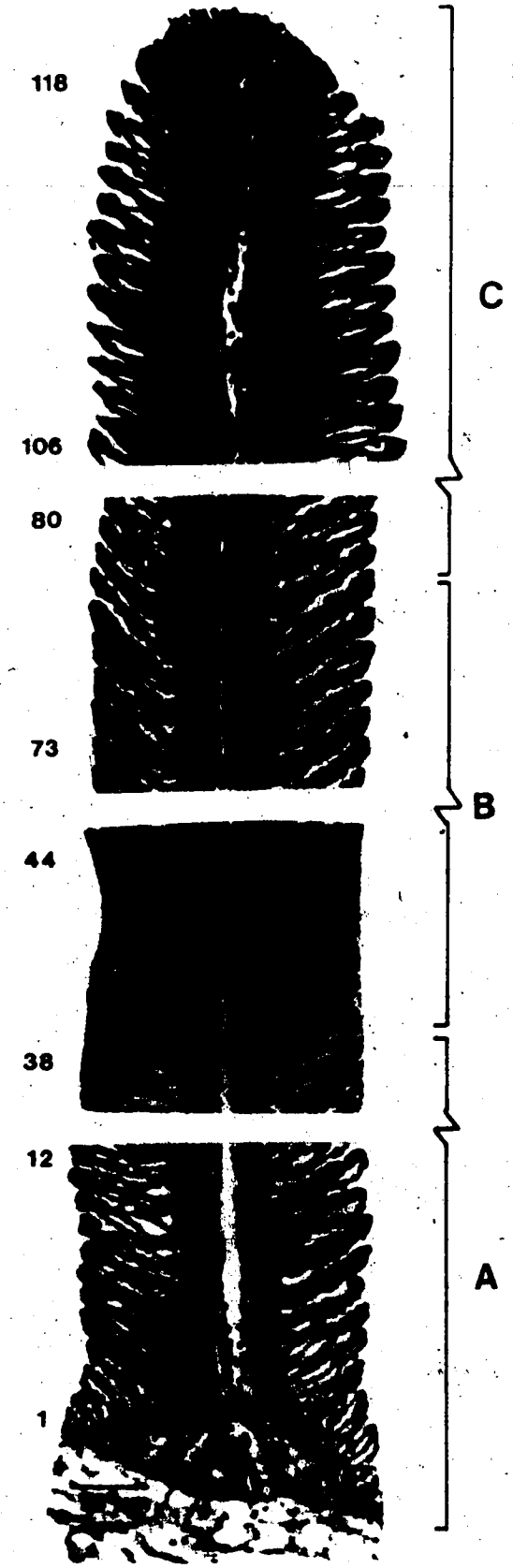


Figure 3. Gill filament of rainbow trout, showing the three regions sampled for morphometric analysis. A - proximal third; B - middle third; C - distal third. The secondary lamellae are numbered.


118
106
80
73
44
38
12
1
C
B
A

curvilinear Merz grid (Weibel, 1973; Hughes and Perry, 1976). The grids were superimposed on the image projected by the microfilm reader. Table 3 summarizes the grids and the magnifications used for each volume and surface area estimated. The two parameters for calculation of a given ratio were measured simultaneously from the same photograph. A minimum of two counts per parameter per photograph were done, each count taken with the photograph orientated 90° from the previous count. If the two counts differed by more than 6%, further counts were taken at 90° intervals until two consecutive counts were within 6% of each other. The various counts were taken only from entire secondary lamellae and the adjacent primary lamellar epithelium. This was in contrast to Hughes and Perry (1976) who measured only portions of the secondary lamellae.

E. Statistical Analyses

LC_{50}

For the LC_{50} determinations, the 4-day and 8-day LC_{50} values and the confidence limits were estimated by the Litchfield and Wilcoxin (1949) method. The slopes of the lines, formed by plotting percent mortality (probability scale) versus measured copper concentration (log scale), and the confidence limits of the slopes were

TABLE 3. Grids and magnifications used to estimate gill parameters

Parameter*	Magnification	Grid Type	Grid Spacing (cm)
V_{BC}	3100x	point	0.8
V_{PS}	3100x	point	0.8
V_{EP}	1540x	point	1.0
$V_{SL'}$	1540x	point	1.0
S_I	1540x	Merz	1.0
S_O	1540x	Merz	1.0

*V = volume, S = surface area, see Figure 2 for description of remaining symbols.

also determined (Litchfield and Wilcoxin, 1949).

Chronic exposure experiment

The data for the physiological parameters (total body water and the plasma concentrations of Cl^-, Na^+, K^+ and glucose) at each copper concentration were analyzed by a Kruskal-Wallis one-way analysis of variance, which included pairwise comparisons between days of exposure, (Marascuilo and McSweeney, 1977) and by a test for non-specific curvilinearity (Nie *et al.*, 1975). The test for curvilinearity was used to determine if a change and a recovery in the data (i.e., a "bump" in the graph) was significant. Because this test may also indicate significance with a square or inverse-square relationship, a "bump" must be present in the graph in order to use the test to determine the significance of a "change and recovery".

Two-way analyses of variance (between concentration and day) could not be reliably used for the physiological parameters measured due to statistical interactions between the two factors (see Sokal and Rohlf, 1969; Nie *et al.*, 1975). Similarly the results of series A and series B could not be pooled due to interactions between the series.

The results for each gill ratio (V_{BC}/V_{PS}, V_{EP}/V_{SL}, S_I/S_O) were analyzed by means of the Hodges-Lehmann Conditional Rank Test which is a non-parametric equivalent

of a two-way anova (Marascuilo and McSweeney, 1977). If significant differences were found with the Hodges-Lehmann test, further analyses were carried out with the Kruskal-Wallis one-way analysis of variance and the test for non-specific curvilinearity.

Correlation coefficients (Spearman's r; Sokal and Rohlf, 1969) were determined between: (i) total body water and each of the plasma ions, (ii) plasma glucose and each of the plasma ions, and (iii) each of the gill ratios and each of the physiological parameters.

RESULTS

I. Median Lethal Concentration (LC_{50}) of Copper to Trout

The 4-day and 8-day LC_{50}'s of copper to rainbow trout were determined as reference indicators of the toxicity of the copper solutions used during the chronic exposure experiments.

The 4-day LC_{50}, based on test C (Figure 4A and Appendix VIIIA), was 148 (139-158; 95% confidence limits) µg/L as total copper and 118 (110-126) µg/L as dissolved copper while the 8-day LC_{50} (Figure 4B and Appendix VIIIA) was 103 (88-120) µg/L as total copper and 87 (77-97) µg/L as dissolved copper. It is unlikely that these values represent a true incipient lethal level (Sprague, 1969) of copper to rainbow trout since mortality was total by the end of the experiment (Appendix VIIIB).

The results of tests A and B were not used in the final determination of the LC_{50}'s due to the problems discussed in Appendix VIIIC.

II. Chronic Exposure to Copper

A. Blood Parameters

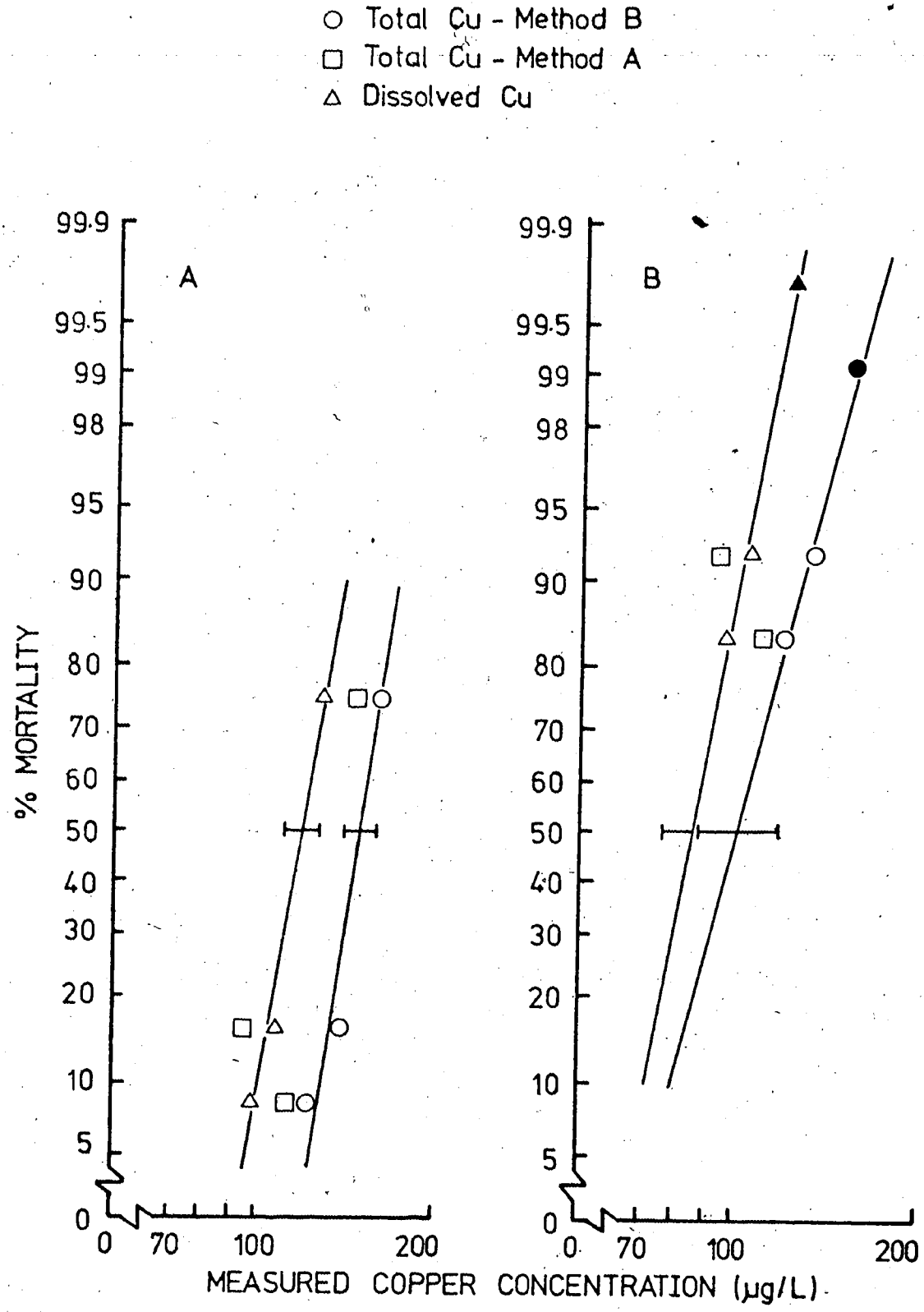
The concentrations of Cl^-, Na^+, K^+ and glucose in the plasma of trout exposed to various concentrations of copper were measured several times over a 64 day period.

Figure 4. Percentage mortality of rainbow trout exposed to various concentrations of copper, test C. Twelve fish were exposed to each concentration of copper. The lines were fitted by eye and the 100% mortality points (▲, ●) were adjusted according to the method described by Litchfield and Wilcoxin (1949). Error bars indicate the 95% confidence limits of the LC_{50} (median lethal concentration).

Total copper was analyzed by two different methods in this test. In Method A, the water samples were acidified before storage while in Method B, the water samples were acidified and then filtered through a 0.45 µm membrane filter before storage. Method A, the method which had been used to analyze copper during the chronic exposure experiments, was considered unreliable due to anomolous results (see Appendix V).

A. Percentage mortality after 96 hours of exposure to copper.

B. Percentage mortality after 192 hours of exposure to copper.


○ Total Cu - Method B
□ Total Cu - Method A
△ Dissolved Cu
A
B
99.9
99.5
99
98
95
90
80
70
60
50
40
30
20
10
5
0
% MORTALITY
0
70
100
200
MEASURED COPPER CONCENTRATION (µg/L)

The data were analyzed with a Kruskal-Wallis one-way analysis of variance and a test for curvilinearity. The data from series A could not be pooled with the data from series B due to differences in blood sampling techniques (Materials and Methods), in pre-treatment of the fish for fin-rot (Table 2) and due to significant interactions between the series as determined by 3-way anovas (Nie *et al.*, 1975). Thus, the results from series A are described separately in Appendix X and, for the purposes of the present study, series A was considered a trial run.

Plasma chloride (Cl^-)

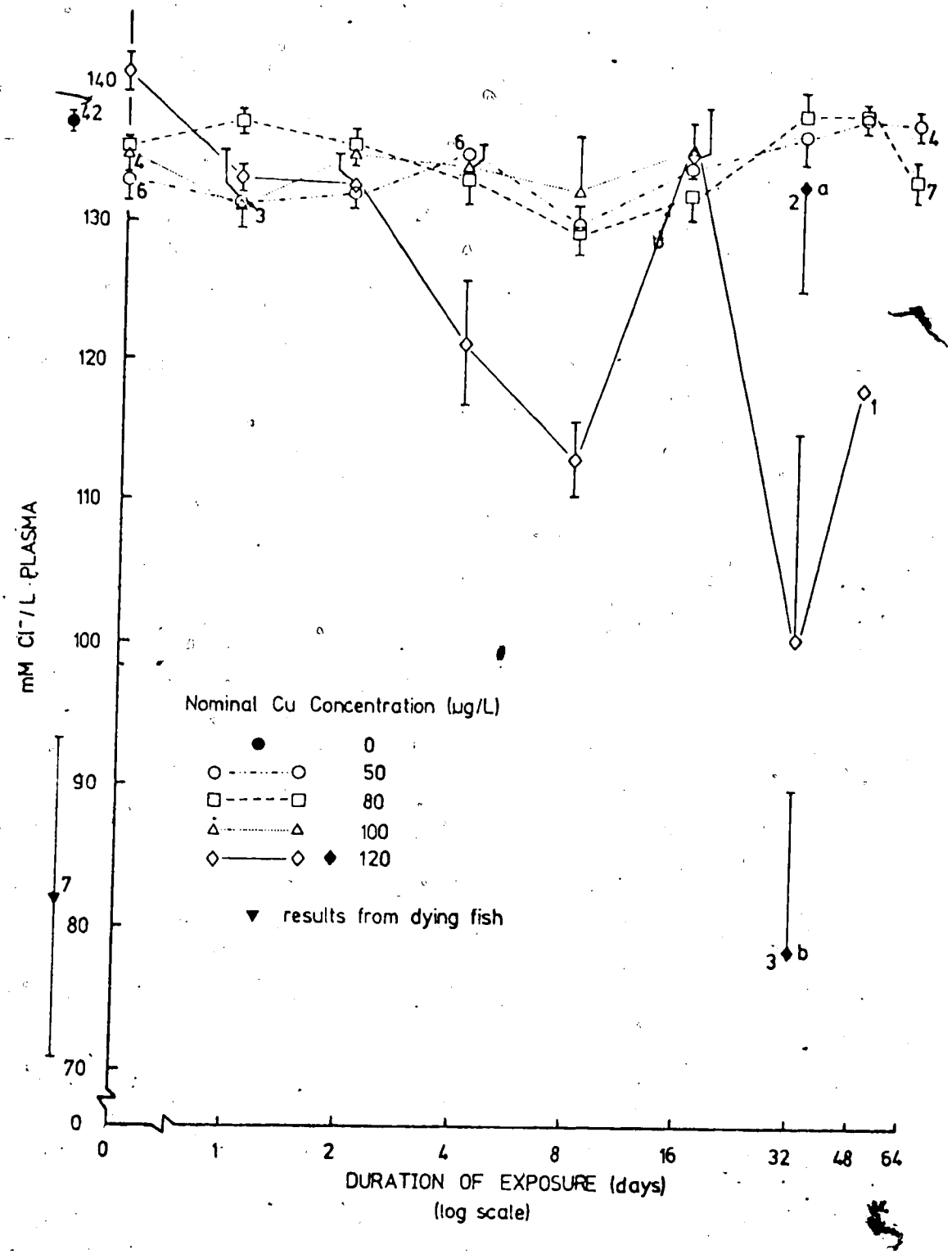
The most significant changes in the plasma Cl^- concentrations over time ($p < 0.01$) were measured from fish exposed to 120 µg/L copper (Table 4). The plasma Cl^- levels steadily declined during the first eight days of exposure and then rose to near the levels measured from the control fish (Figure 5). Some fish were not able to recover as shown by the continued decline in plasma Cl^- values for three fish sampled on day 32 (32b) which had plasma Cl^- values close to those measured from dying fish (Figure 5). The plasma Cl^- levels measured from fish sampled on day 8 and from the three fish identified as the day 32b samples were significantly different ($p < 0.05$) from the levels measured from fish sampled on day 0 (Table 4).

TABLE 4. Results of statistical tests to determine significant differences over time in the plasma Cl^- concentrations of trout chronically exposed to copper, series B.

Statistical Test	nominal Cu concentration (µg/L) 0	50	80	100	120 with all day 32 results	120 with day 32a results[2]	120 with day 32b results[2]
Kruskal-Wallis One-Way Analysis of Variance	NS	*	*	NS	**	**	**
Days Significantly Different ($p < 0.05$)[1]	NS	NS	NS	NS	NS	0+8	0+32b
Test for Curvilinearity	NS	NS	*	NS	NS	**	**

[1] Pairwise comparisons between days based on Kruskal-Wallis mean ranks,
[2] See Figure 5 for definition of day 32a and day 32b results,
* ($p < 0.05$), ** ($p < 0.01$).

Figure 5. Plasma chloride values (mean ± S.E.M.) of trout chronically exposed to copper, series B. Except where indicated each point represents the mean value for 5 fish. The point indicated at (a) represents the mean value of the fish sampled on day 32 with plasma Cl^- and Na^+ values near the control mean. The point indicated at (b) represents the mean value of the fish sampled on day 32 with plasma Cl^- and Na^+ values well below the control mean.


mM Cl⁻/L PLASMA
140
130
120
110
100
90
80
70
0
0
1
2
4
8
16
32
48
64
DURATION OF EXPOSURE (days)
(log scale)
Nominal Cu Concentration (µg/L)
0
50
80
100
120
results from dying fish
a
b

Though no overt sign of impending death was evident from the fish sampled during the 120 µg/L copper exposure, the steady decline of the plasma Cl^- concentrations may have been partially the result of sampling fish that would not have recovered from the copper treatment. This is suspected due to the fact that most of the deaths during the 120 µg/L Cu exposure occurred within the first 15 days (Appendix IX). Thus, the apparent recovery may be due to a lack of a severe effect on most fish that survived the initial 15 days of exposure.

However, statistically significant ($p < 0.05$) changes in plasma Cl^- levels over time were also observed in fish exposed to 50 and 80 µg/L Cu (Table 4). The lowest levels were measured from the fish sampled on day 8 which illustrates some similarity in pattern to the changes at 120 µg/L Cu. Since very little mortality occurred at these concentrations (Appendix IX), the decline and recovery in the plasma Cl^- values appear to illustrate the ability of the fish to adapt to copper.

Plasma sodium (Na^+)

At the 120 µg/L copper exposure, the plasma sodium of the fish changed in a manner similar to the changes observed in the plasma Cl^- levels. A steady decline in values occurred during the first eight days of exposure, followed by a recovery and a split in values after 32

days of exposure (Figure 6). The changes over time were highly significant ($p < 0.01$) but the pairwise comparisons between days could not differentiate which individual days were significantly different (Table 5).

As with the plasma Cl^- values, part of the initial decline in plasma Na^+ values in fish exposed to 120 µg/L Cu may have been due to the sampling of fish that would not have recovered from the copper exposure.

No significant changes in plasma Na^+ values over time were found in fish exposed to the lower concentrations of copper (Table 5). This indicates that, compared with the changes in plasma Cl^- levels, the levels of plasma Na^+ are better controlled by the adaptive mechanisms of the fish.

Plasma potassium (K^+)

The levels of potassium in the plasma of the trout were quite variable (Figure 7). In the fish exposed to 120 µg/L Cu a significant decline in plasma K^+ over time was noted when the fish indicated at day 32b were considered (Table 6). No significant changes over time were noted when the fish indicated at day 32a were considered. However, at day 16, the K^+ value of fish exposed to 120 µg/L Cu differed significantly from the K^+ value of the control fish (Kruskal-Wallis, $p < 0.05$) while no differences were noted between the two groups at days 0 to 8. Thus, the increase in K^+ for the fish

Figure 6. Plasma sodium values (mean ± S.E.M.) of trout chronically exposed to copper, series B. Except where indicated each point represents the mean value for 5 fish. The point indicated at (a) represents the mean value of the fish sampled on day 32 with plasma Cl^- and Na^+ values near the control mean. The point indicated at (b) represents the mean value of the fish sampled on day 32 with plasma Cl^- and Na^+ values well below the control mean.

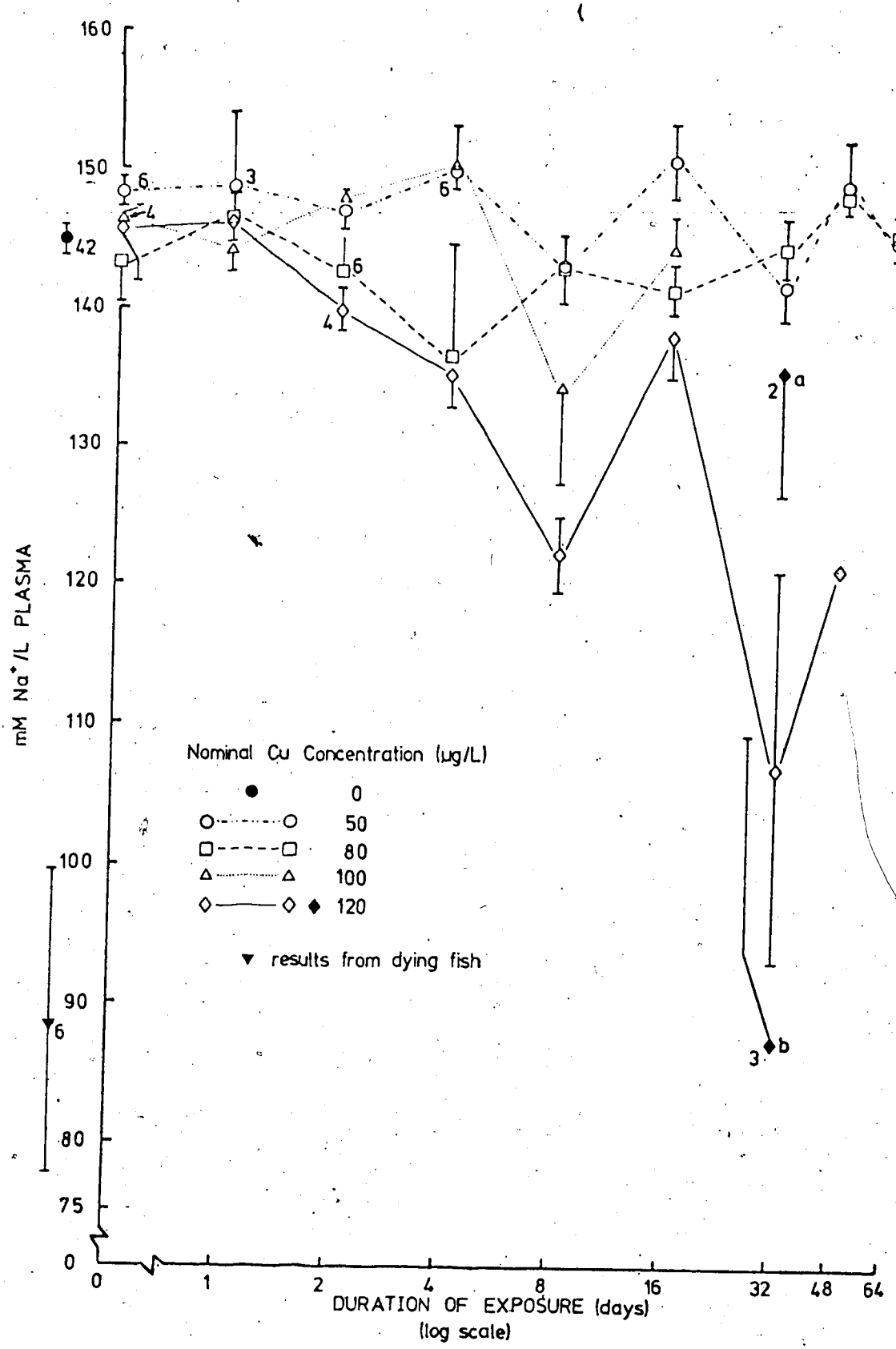

160
150
140
130
120
110
100
90
80
75
0
42
mM Na⁺/L PLASMA
Nominal Cu Concentration (µg/L)
0
50
80
100
120
results from dying fish
0
1
2
4
8
16
32
48
64
DURATION OF EXPOSURE (days)
(log scale)
a
b

TABLE 5. Results of statistical tests to determine significant differences over time in the plasma Na^+ concentrations of trout chronically exposed to copper, series B.

Statistical Test	nominal Cu concentration (µg/L)						
	0	50	80	100	120 with all day 32 results	120 [2] with day 32a results	120 [2] with day 32b results
Kruskal-Wallis One-Way Analysis of Variance	NS	NS	NS	NS	**	**	**
Days Significantly Different ($p < 0.05$) [1]	NS	NS	NS	NS	NS	NS	NS
Test for Curvilinearity	NS	NS	NS	NS	NS	**	**

[1]Pairwise comparisons between days based on Kruskal-Wallis mean ranks,

[2]See Figure 6 for definition of day 32a and day 32b results,

*($p < 0.05$), **($p < 0.01$).

TABLE 6. Results of statistical tests to determine significant differences over time in the plasma K^+ concentrations of trout chronically exposed to copper, series B.

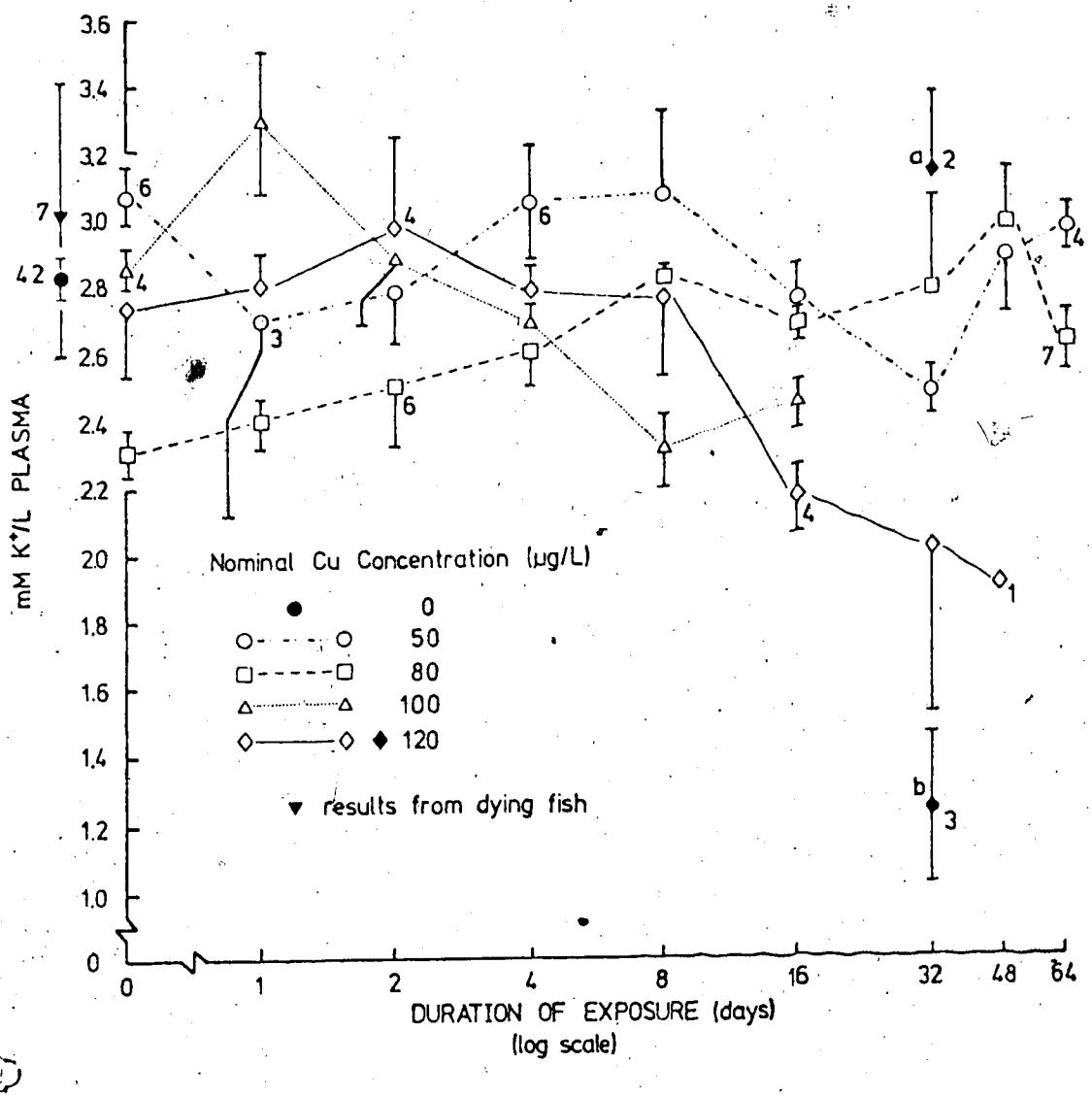
Statistical Test	nominal Cu concentrations (µg/L)						
	0	50	80	100	120 with all day 32 results	120 [2] with day 32a results	120 [2] with day 32b results
Kruskal-Wallis One-Way Analysis of Variance	NS	NS	*	**	NS	NS	**
Days Significantly Different ($p < 0.05$) [1]	NS	NS	NS	NS	NS	NS	NS
Test for Curvilinearity	NS	NS	NS	*	NS	NS	*

[1] Pairwise comparisons between days based on Kruskal-Wallis mean ranks,

[2] See Figure 7 for definition of day 32a and day 32b results,

* ($p < 0.05$), ** ($p < 0.01$).

Figure 7. Plasma potassium values (mean ± S.E.M.) of trout chronically exposed to copper, series B. Except where indicated each point represents the mean value for 5 fish. The point indicated at (a) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ values near the control mean. The point indicated at (b) represents the fish sampled on day 32 which had plasma Cl^- and Na^+ values well below the control mean.


mM K⁺/L PLASMA
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
0
1
2
4
8
16
32
48
64
DURATION OF EXPOSURE (days)
(log scale)
Nominal Cu Concentration (µg/L)
0
50
80
100
120
results from dying fish
a
b

indicated at day 32a may be indicative of a recovery even though no significant changes over time were noted.

Significant changes in plasma K^+ over time were also noted in fish exposed to 100 µg/L Cu. Whether or not the slight increase in values at day 16 was indicative of a recovery could not be determined.

A significant change in plasma K^+ concentration over time was also found for the 80 µg/L Cu-exposed fish. However, the initial K^+ concentration (at day 0) was significantly lower ($p < 0.05$) than the concentration measured from the control fish and the K^+ concentration during the 80 µg/L Cu exposure increased towards the control value (Figure 7). Thus, it is unlikely that the changes noted during the 80 µg/L Cu exposure were due to copper.

Plasma glucose

A marked increase in the plasma concentration of glucose was measured from trout during the first eight days of exposure to 120 µg/L Cu (Figure 8, Table 7), which indicates an increase in stress (Mazeaud *et al.*, 1977) over that time period. The pattern of change in the plasma glucose levels was opposite the pattern found for the plasma levels of Cl^- and Na^+ (Figures 5 and 6). Thus, it appears that a decline in the plasma concentrations of Cl^- and Na^+ was associated with an increase in stress

Figure 8. Plasma glucose values (mean ± S.E.M.) of trout chronically exposed to copper, series B. Except where ind[illegible]d each point represents the mean value f[illegible]. The point indicated at (a) repres[illegible]ean value of the fish sampled on day 32 w[illegible] had plasma Cl^- and Na^+ values near the control mean. The point indicated at (b) represents the fish sampled on day 32 which had plasma Cl^- and Na^+ values well below the control mean.

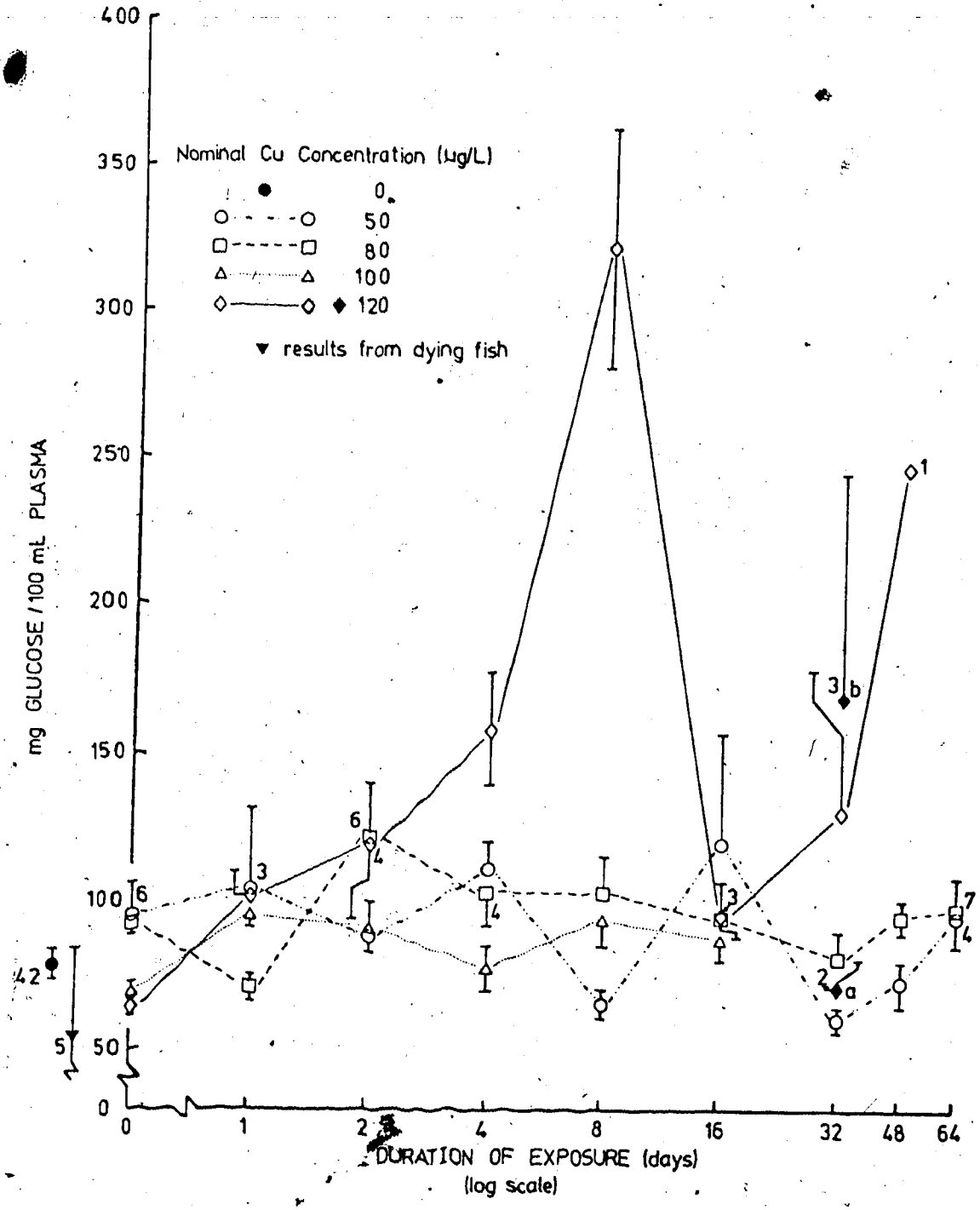

Nominal Cu Concentration (µg/L)
0
50
80
100
120
▼ results from dying fish
mg GLUCOSE / 100 mL PLASMA
400
350
300
250
200
150
100
50
0
0
1
2
4
8
16
32
48
64
DURATION OF EXPOSURE (days)
(log scale)

TABLE 7. Results of statistical tests to determine significant differences over time in the plasma glucose concentrations of trout chronically exposed to copper, series B.

Statistical Test	nominal Cu concentrations (µg/L)						
	0	50	80	100	120 with all day 32 results	120 with day 32a results[2]	120 with day 32b results[2]
Kruskal-Wallis One-Way Analysis of Variance	*	**	NS	NS	**	**	**
Days Significantly Different ($p < 0.05$)[1]	NS	NS	NS	NS	0+8	0+8	0+8
Test for Curvilinearity	NS	NS	NS	NS	**	**	**

[1] Pairwise comparisons between days based on Kruskal-Wallis mean ranks,

[2] See Figure 8 for definitions of day 32a and day 32b results,

* ($p < 0.05$), ** ($p < 0.01$).

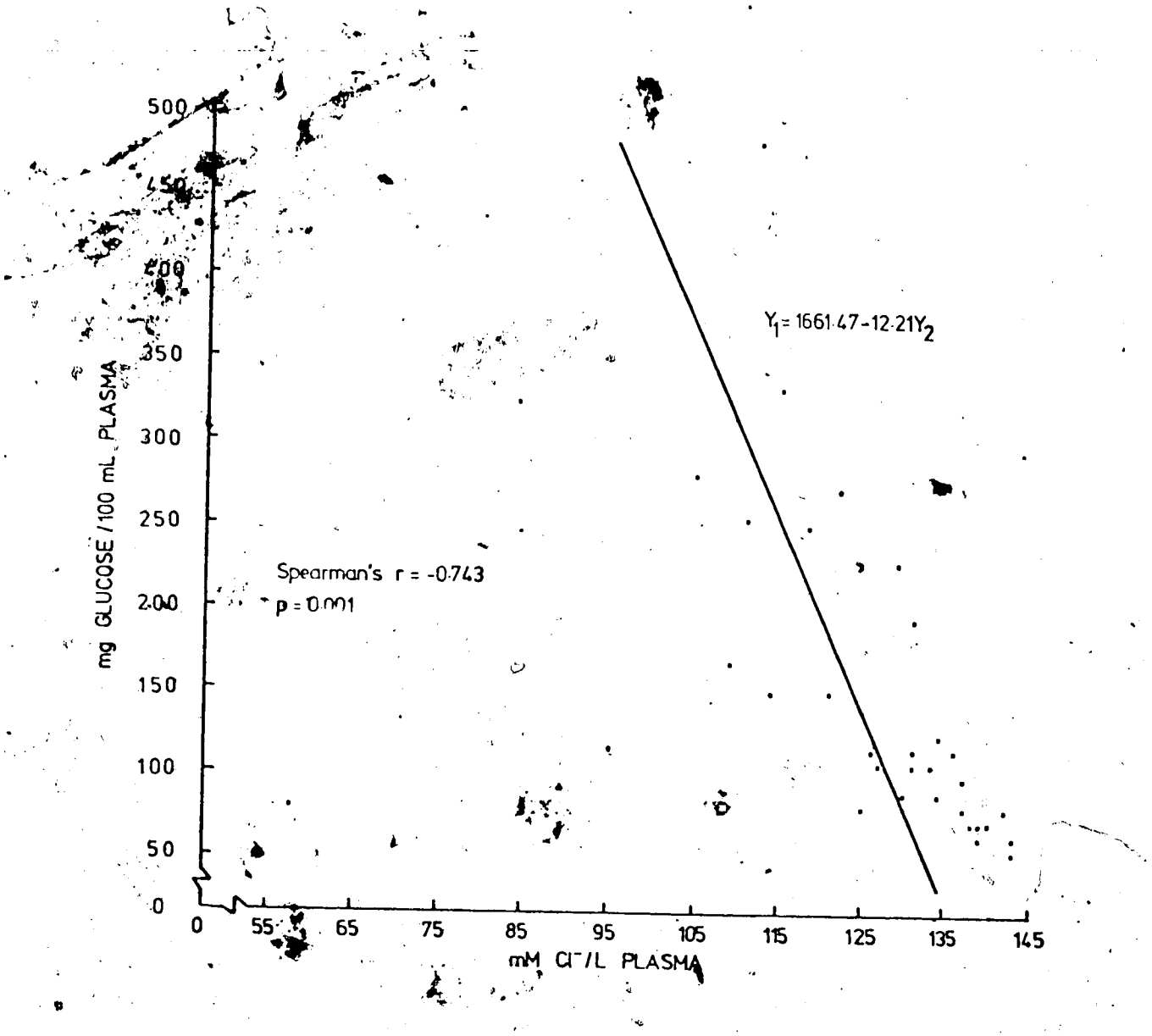
and that a return of the plasma concentrations of Cl^- and Na^+ to near control levels was associated with a decrease in the stress experienced by the fish. That association is further supported by highly significant negative correlations ($p = 0.001$) between the plasma concentration of glucose and both the plasma concentrations of Cl^- and Na^+ (Figures 9, 10). Similarly, negative, but less significant correlations ($p < 0.05$) between plasma glucose and plasma Cl^- occurred at both the 80 and 100 µg/L copper exposures (Spearman's r = -0.220 and -0.277, respectively) and with some of the fish exposed to copper during series A (Appendix X - Table X-3).

B. Total Body Water

The percentage of water in whole fish was measured several times following exposure to copper as another indicator of changes in osmoregulation by the trout.

No significant changes in percent body water were found during any of the copper exposures. However, significant negative correlations ($p < 0.05$) were found between the percentage of body water and both the plasma concentrations of Cl^- and Na^+ in fish exposed to 120 µg/L Cu (Figures 11, 12), indicating that a reduction in the concentration of these ions is associated with an increase in the amount of water in the fish. Even

Figure 9. Scatter plot of plasma glucose versus plasma chloride concentrations of trout chronically exposed to 120 µg/L Cu, series B. The line drawn through the plot represents the major axis as calculated according to Sokal and Rohlf (1969). The 95% confidence limits of the slope are: (-7.055, -44.444). Y_1 = plasma glucose concentration, Y_2 = plasma chloride concentration.


500
450
400
350
300
250
200
150
100
50
0
mg GLUCOSE / 100 mL PLASMA
Spearman's r = -0.743
p = 0.001
$Y_1 = 1661.47 - 12.21Y_2$
0
55
65
75
85
95
105
115
125
135
145
mM Cl⁻/L PLASMA

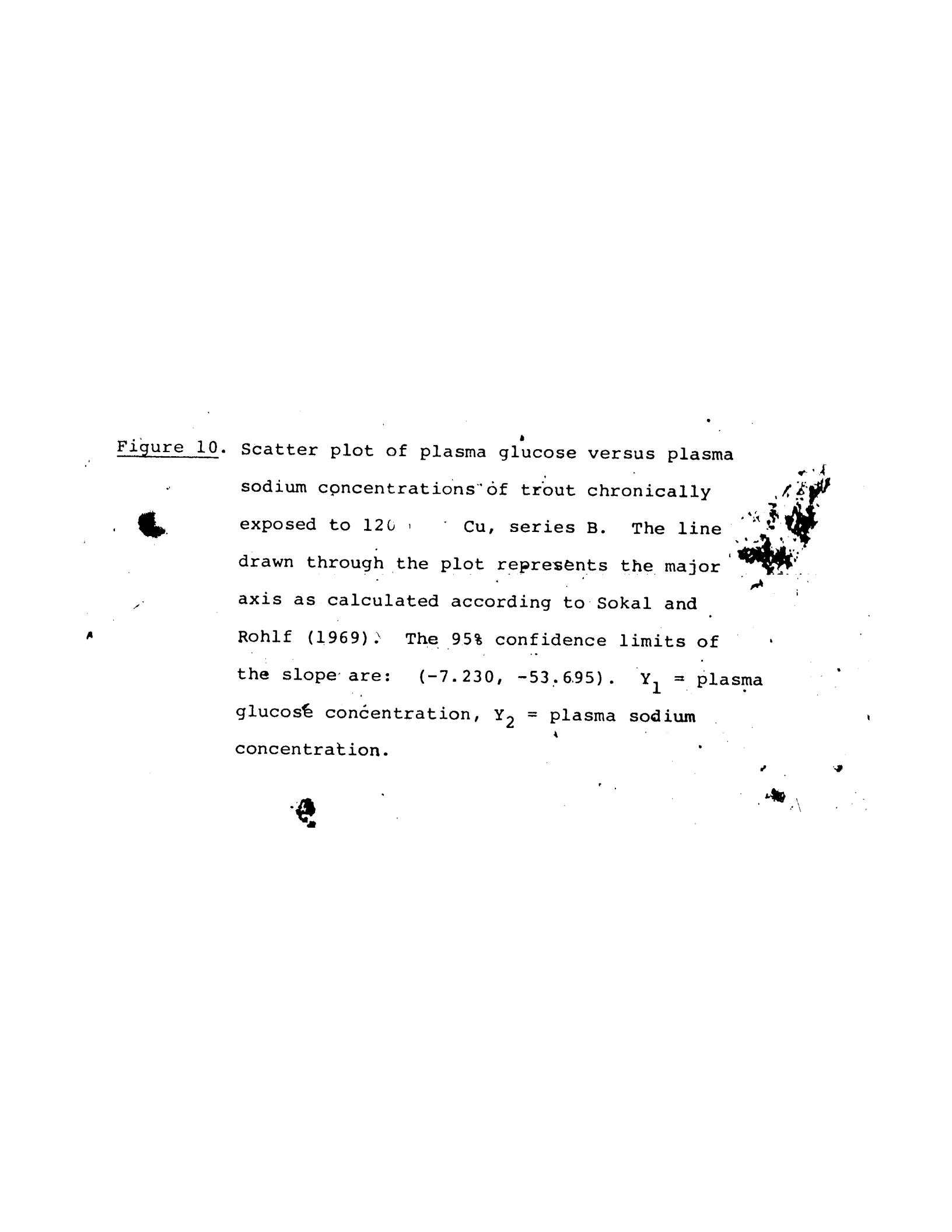
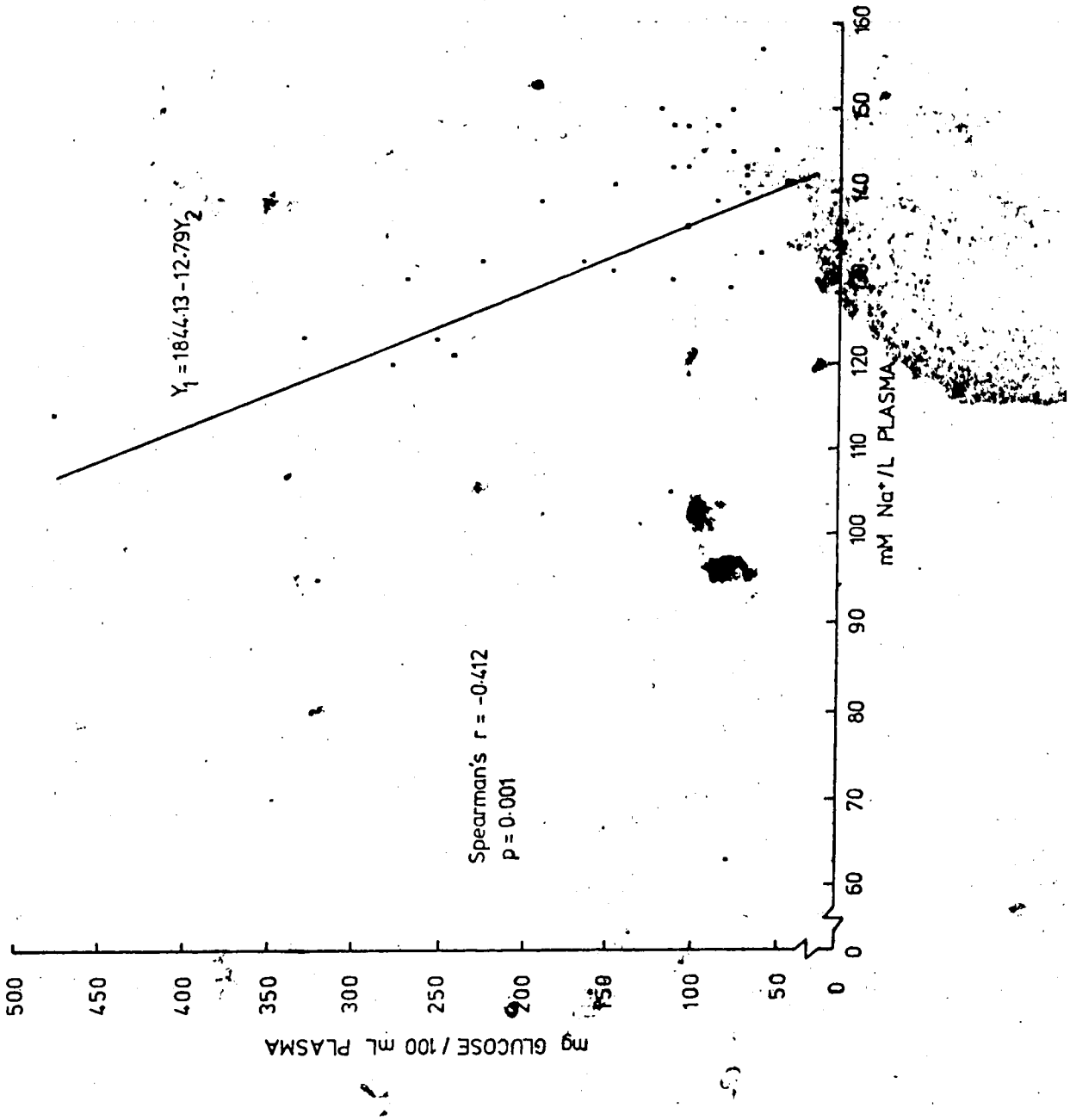


Figure 10. Scatter plot of plasma glucose versus plasma sodium concentrations of trout chronically exposed to 120 Cu, series B. The line drawn through the plot represents the major axis as calculated according to Sokal and Rohlf (1969). The 95% confidence limits of the slope are: (-7.230, -53.695). Y_1 = plasma glucose concentration, Y_2 = plasma sodium concentration.


$Y_1 = 1844.13 - 12.79Y_2$
Spearman's r = -0.412
p = 0.001
mg GLUCOSE / 100 mL PLASMA
mM Na^+/L PLASMA
0
50
100
150
200
250
300
350
400
450
500
60
70
80
90
100
110
120
130
140
150
160

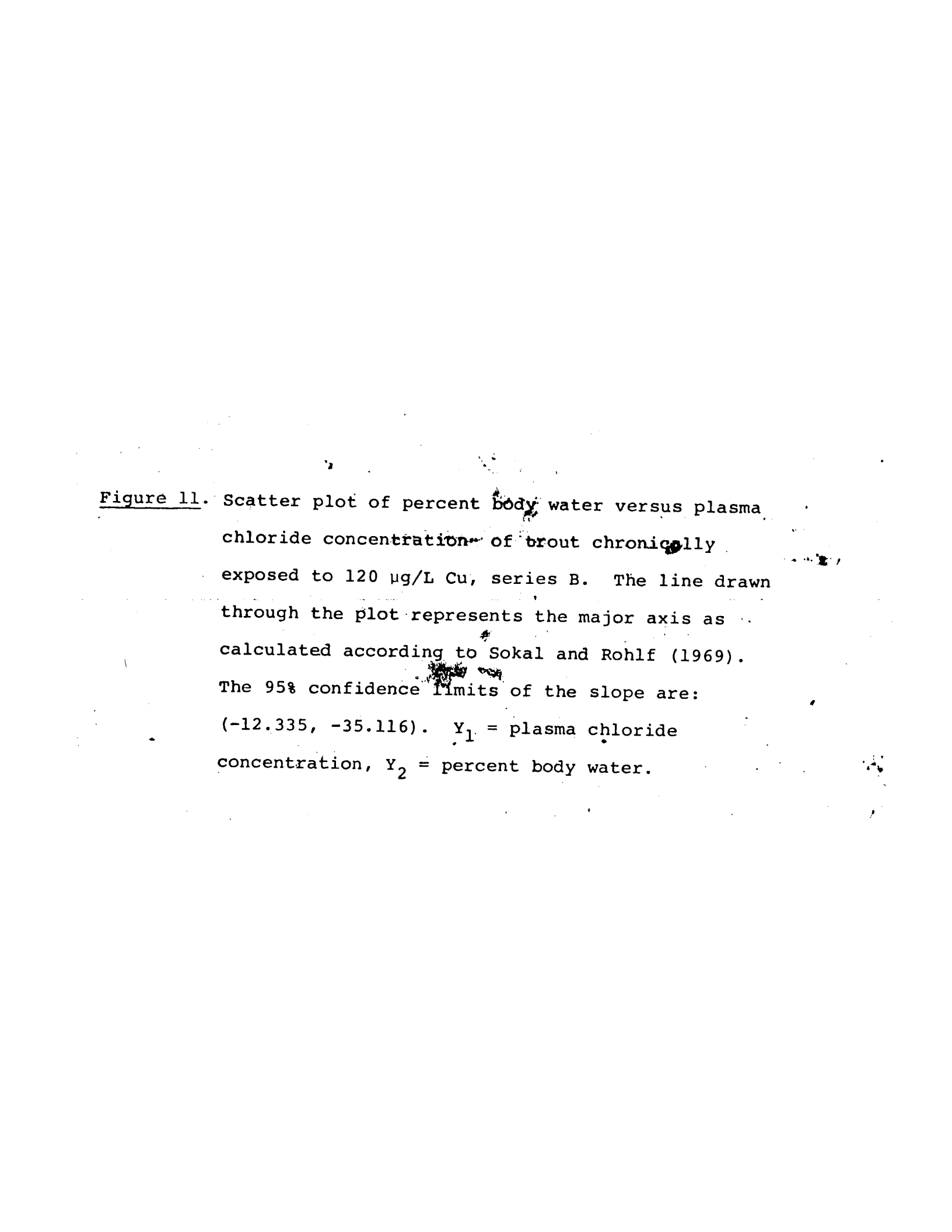


Figure 11. Scatter plot of percent body water versus plasma chloride concentration of trout chronically exposed to 120 µg/L Cu, series B. The line drawn through the plot represents the major axis as calculated according to Sokal and Rohlf (1969). The 95% confidence limits of the slope are: (-12.335, -35.116). Y_1 = plasma chloride concentration, Y_2 = percent body water.

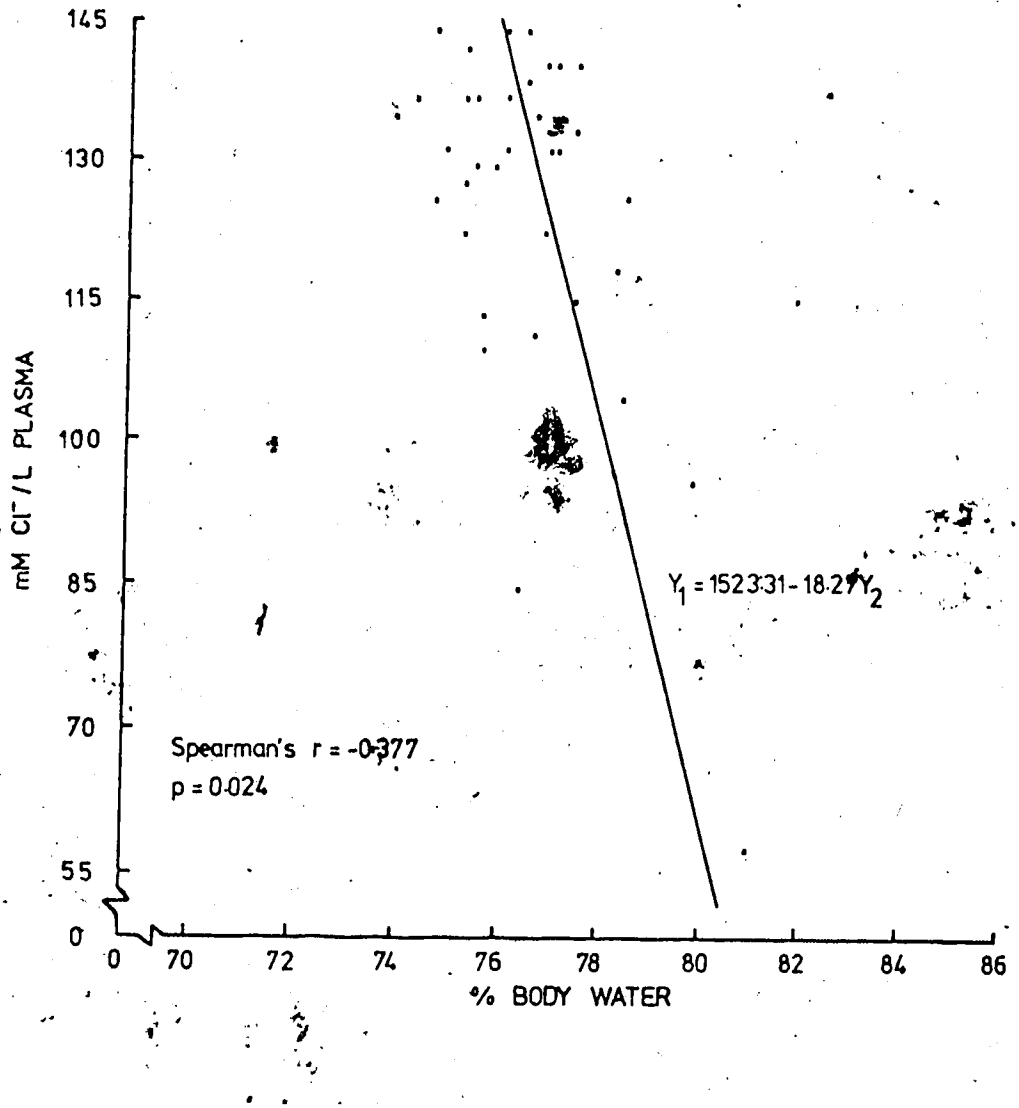
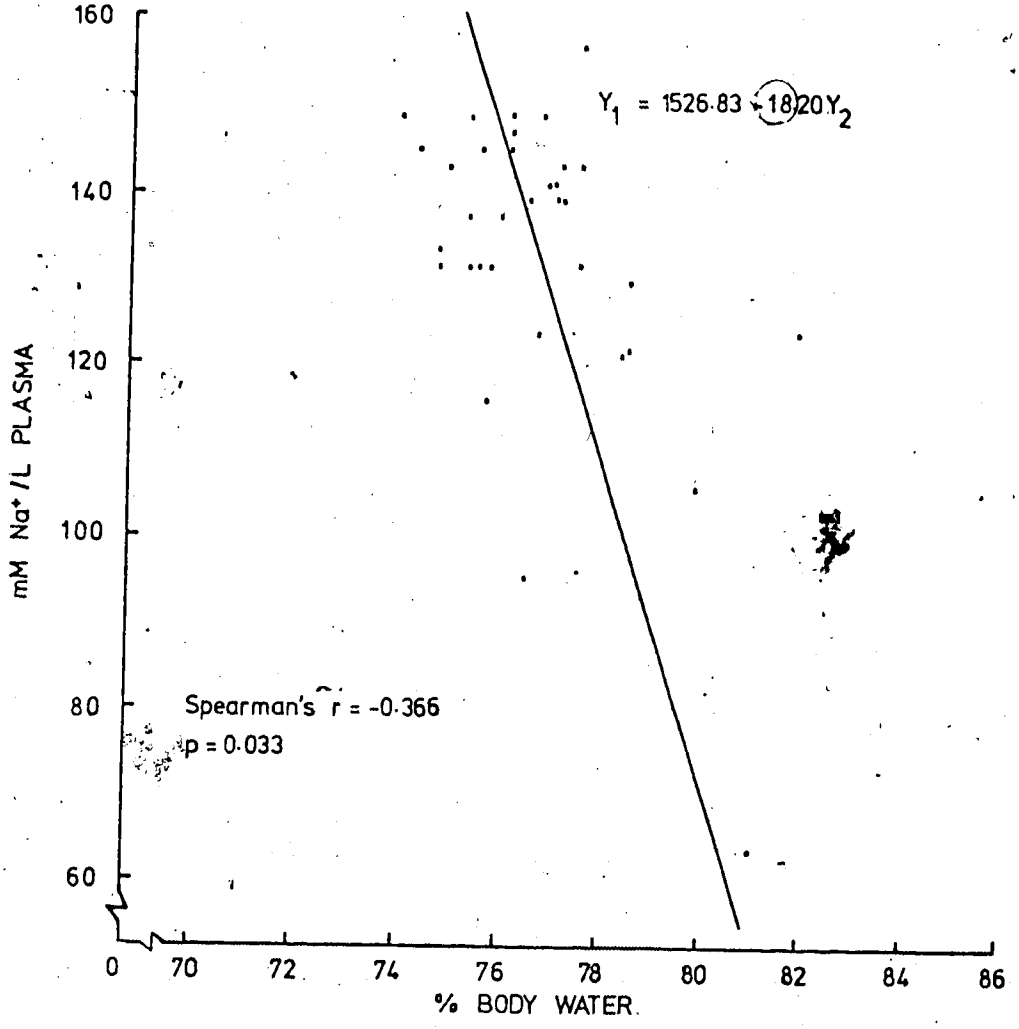

145
130
115
100
85
70
55
0
mM Cl⁻/L PLASMA
0
70
72
74
76
78
80
82
84
86
% BODY WATER
Y1 = 152·31 - 18·27Y2
Spearman's r = -0·377
p = 0·024

Figure 12. Scatter plot of percent body water versus plasma sodium concentration of trout chronically exposed to 120 µg/L Cu, series B. The line drawn through the plot represents the major axis as calculated according to Sokal and Rohlf (1969). The 95% confidence limits of the slope are: (-12.073, -36.840). Y_1 = plasma sodium concentration, Y_2 = percent body water.


160
140
120
100
80
60
mM Na⁺/L PLASMA
$Y_1 = 1526.83 - 18.20 Y_2$
Spearman's r = -0.366
p = 0.033
0
70
72
74
76
78
80
82
84
86
% BODY WATER

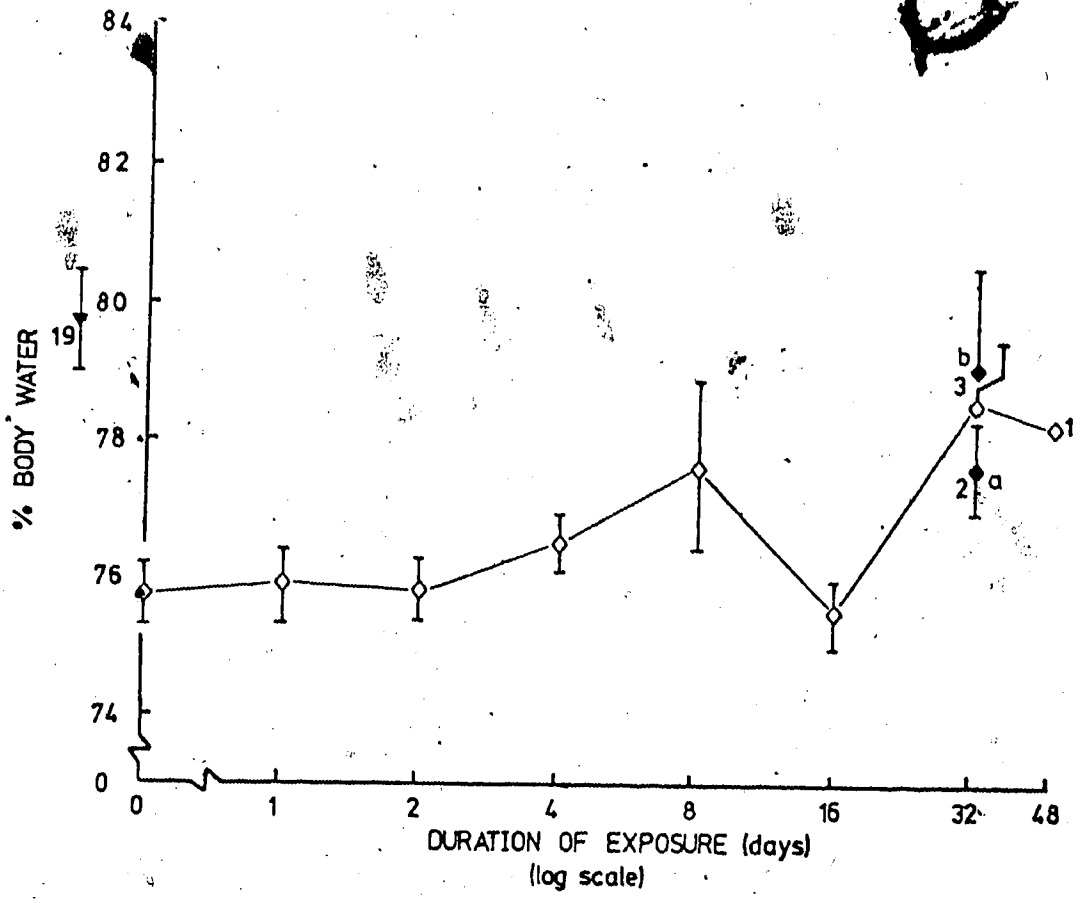
though no statistically significant changes occurred in total body water over time at the 120 µg/L Cu exposure, the changes did follow a pattern (Figure 13) opposite that found for plasma levels of Cl^- and Na^+ (Figures 5,6). Thus, it appears that an increase in the percentage of water in the fish may have diluted the plasma. The probable dilution of the plasma was further shown by the high percentage of water in fish sampled while dying (Figure 13) compared to the low values obtained from the same fish for plasma levels of Cl^-, Na^+ and glucose (Figures 5,6,8).

The fact that a dilution of the K^+ concentration was not found in the plasma of dying fish (Figure 8) could be due to the release of K^+ from the erythrocytes as the fish were dying. Since erythrocytes contain a very high proportion of K^+ compared to the plasma (Wilson, 1972), partial hemolysis of these cells could easily increase the plasma K^+ concentration.

C. Histological Parameters

The following ratios were determined from the gills of fish exposed for up to 32 days to the 0 and the 120 µg/L copper concentrations (series B): (i) volume of the blood channel spaces/volume of the entire pillar system (V_{BC}/V_{PS}), (ii) volume of the epithelial tissue in the gill lamellae/volume of the entire secondary

Figure 13. Percentage of water (mean ± S.E.M.) in trout chronically exposed to 120 µg/L Cu, series B. Except where noted each point represents the mean value of 5 fish. The point at (a) represents the fish sampled on day 32 which had plasma Cl^- and Na^+ values near the control mean. The point at (b) represents the fish sampled on day 32 which had plasma Cl^- and Na^+ values well below the control mean. (▲) represents the mean value from dying fish.


84
82
80
78
76
74
0
% BODY WATER
19
b
3
1
2
a
0
1
2
4
8
16
32
48
DURATION OF EXPOSURE (days)
(log scale)

lamellae including the adjacent epithelium of the primary lamella and excluding non-tissue spaces ($V_{EP}/V_{SL'}$) and (iii) surface area of the pillar system (which is equivalent to the surface area of the epithelial basement membrane adjacent to the pillar system)/outer surface area of the gill epithelia (S_I/S_O).

The results obtained from the three regions of the gill filaments sampled (see Figure 3) were compared by means of t-tests to determine if the ratios obtained from one region of the filaments differed significantly from the ratios obtained from the other regions (Appendix XI). Few significant differences were found between the regions of the gill filaments for the V_{BC}/V_{PS} and the $V_{EP}/V_{SL'}$ ratios. Therefore, for each of those two ratios, the values from the three regions were averaged for each fish. Then, the average values were each used as a single value for further statistical analyses. A high percentage of the S_I/S_O ratios from region C (the distal third of the gill filaments) differed significantly from the ratios found from regions A and B (Appendix XI). Thus, the S_I/S_O ratios from region C were analyzed separately while the ratios from regions A and B were averaged.

The results for each ratio were analyzed by means of the Hodges-Lehmann Conditional Rank Test which is a non-parametric equivalent of a two-way anova (Marascuilo and McSweeney, 1977). Where significant differences were

found with the Hodges-Lehmann Test, further analyses were carried out with a Kruskal-Wallis one-way analysis of variance and a test for curvilinearity.

The separation of epithelia from the basement membrane of secondary lamellae, a phenomenon commonly reported to be an effect of exposure to pollutants (e.g., McFadden, 1965; Bilinski and Jones, 1973; Daye and Garside, 1976; Skidmore and Tovell, 1972) could not be examined in this study because separation of the epithelia occurred in over 90% of the samples from both the control and the 120 µg/L Cu-exposed fish. An attempt to discover the cause of that separation is described in Appendix XII.

V_{BC}/V_{PS}

No significant differences were found in the proportion of blood channel spaces in the pillar system over the 32 days of exposure to copper or between the 0 and the 120 µg/L copper exposures. However, a trend towards proportionally larger blood channel spaces occurred in the gills of the fish exposed to copper (Figure 14).

A significant negative correlation ($p < 0.05$) was found between V_{BC}/V_{PS} and the concentration of plasma potassium (Table 8) for both the 0 and the 120 µg/L Cu exposures. The biological significance of this correlation is not known.

Figure 14. Ratio between the volume of the blood channel spaces and the volume of the pillar system (V_{BC}/V_{PS}) of gill lamellae (mean ± S.E.M.) from trout chronically exposed to 0 and to 120 µg/L Cu. Except where noted, each point represents the mean value of 5 fish. The point indicated by (a) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ values near the control mean. The point indicated by (b) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ values well below the control mean.

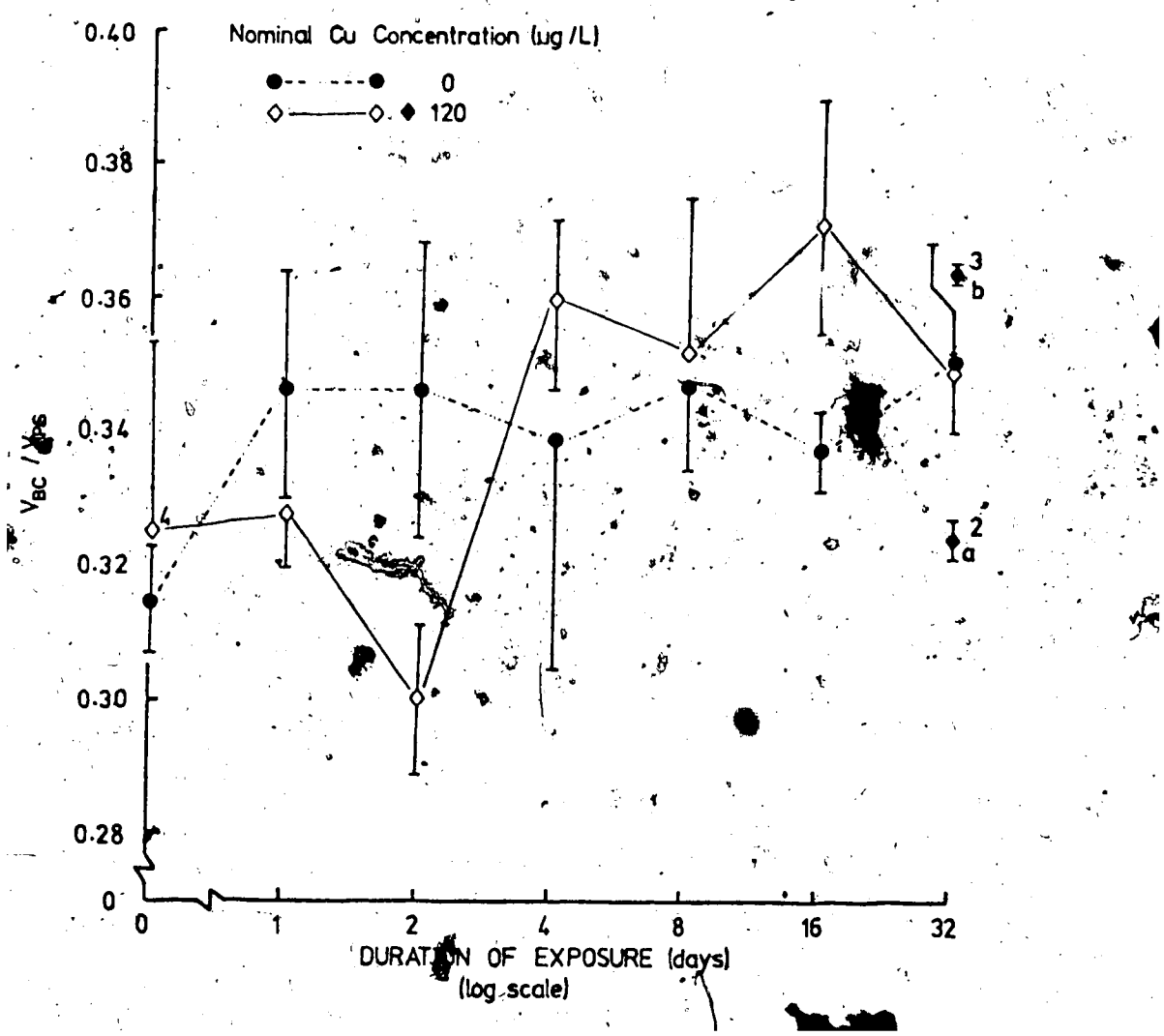

Nominal Cu Concentration (ug/L)
0
120
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0
V_{BC} / V_{BS}
4
3
b
2
a
0
1
2
4
8
16
32
DURATION OF EXPOSURE (days)
(log scale)

TABLE 8. Correlation coefficients (Spearman's r) between V_{BC}/V_{PS} and the physiological parameters of trout ...ically exposed to copper, series B

Physiological Parameter	V_{BC}/V_{PS}[1] Exposure		
	0 µg/L Cu	120 µg/L Cu	0 and 120 µg/L Cu combined
Plasma Chloride (mM/L)	NS		NS
Plasma Sodium (mM/L)	NS	NS	NS
Plasma Potassium (mM/L)	* (-0.409)[2]	* (-0.379)	** (-0.380)
Plasma Glucose (mg/100 mL)	NS	NS	NS

*$p < 0.05$, **$p < 0.01$,

[1]Volume of the blood channel spaces (V_{BC}) relative to the volume of the pillar system (V_{PS}) of the secondary lamellae of trout gills.

[2]Spearman's r indicated in brackets where significant.

V_{EP}/V_{SL}

The proportion of epithelia relative to the total secondary lamellar volume (including the epithelia of the primary lamella adjacent to the secondary lamellae) of fish exposed to 120 µg/L copper was significantly lower than that of the 0 µg/L Cu-exposed fish (Table 9). Since the values determined for fish sampled on day 0 were much lower in the group used for the 120 µg/L Cu exposure than for the 0 µg/L Cu exposure (Figure 15), it appears unlikely that most of the overall difference was due to copper.

However, the changes in V_{EP}/V_{SL} over time did differ between the two concentrations (Figure 15). The control fish showed very little change over time while the 120 µg/L copper-exposed fish showed significant change ($p < 0.05$), with the lowest values after 4 days of exposure.

That changes in the proportion of epithelia in trout gills may be associated with changes in osmoregulatory ability is indicated by significant correlations between V_{EP}/V_{SL} and the plasma concentrations of Cl^- and Na^+ as well as the percent body water (Table 10). Those correlations did not occur when the fish of the 0 and the 120 µg/L Cu exposures were considered separately, but that may be due to the fact that the maximum change

TABLE 9. Results of statistical tests to determine significant differences in $V_{EP}/V_{SL'}$[1] in trout chronically exposed to copper, series B. Comparisons between concentrations of exposure and between days of exposure.

Statistical Test	Independent Variable: Concentration	Independent Variable: Day
Hodges-Lehmann Conditional Rank Test	**	*
Groups Significantly[2] Different ($p < 0.05$)	0 + 120	NS

Statistical Test	Nominal Cu Concentration (µg/L): 0	120 with all day 32 results	120 with[3] day 32a results	120 with[3] day 32b results
Kruskal-Wallis Analysis of Variance	NS	NS	NS	*
Days Significantly[2] Different ($p < 0.05$)	NS	NS	NS	NS
Test for Curvilinearity	NS	*	NS	*

* $p < 0.05$, ** $p < 0.01$, [1]Volume of the gill epithelia (V_{EP}) relative to the volume of the secondary lamellae including the adjacent epithelia of the primary lamella ($V_{SL'}$),

[2]Pairwise comparisons based on mean ranks, [3]See Figure 15 for definition of day 32a and day 32b results.

Figure 15. Ratio between the volume of epithelial tissue in the gill and the volume of the entire secondary lamellae including the adjacent epithelia of the primary lamella (V_{EP}/V_{SL}) (mean ± S.E.M.). The ratios were determined from trout chronically exposed to 0 and to 120 µg/L Cu. Except where noted each point represents the mean value of 5 fish. The point indicated at (a) represents the mean value from the fish sampled on day 32 that had plasma Cl^- and Na^+ values near the control mean. The point indicated at (b) represents the mean value from the fish sampled on day 32 that had plasma Cl^- and Na^+ values well below the control mean.

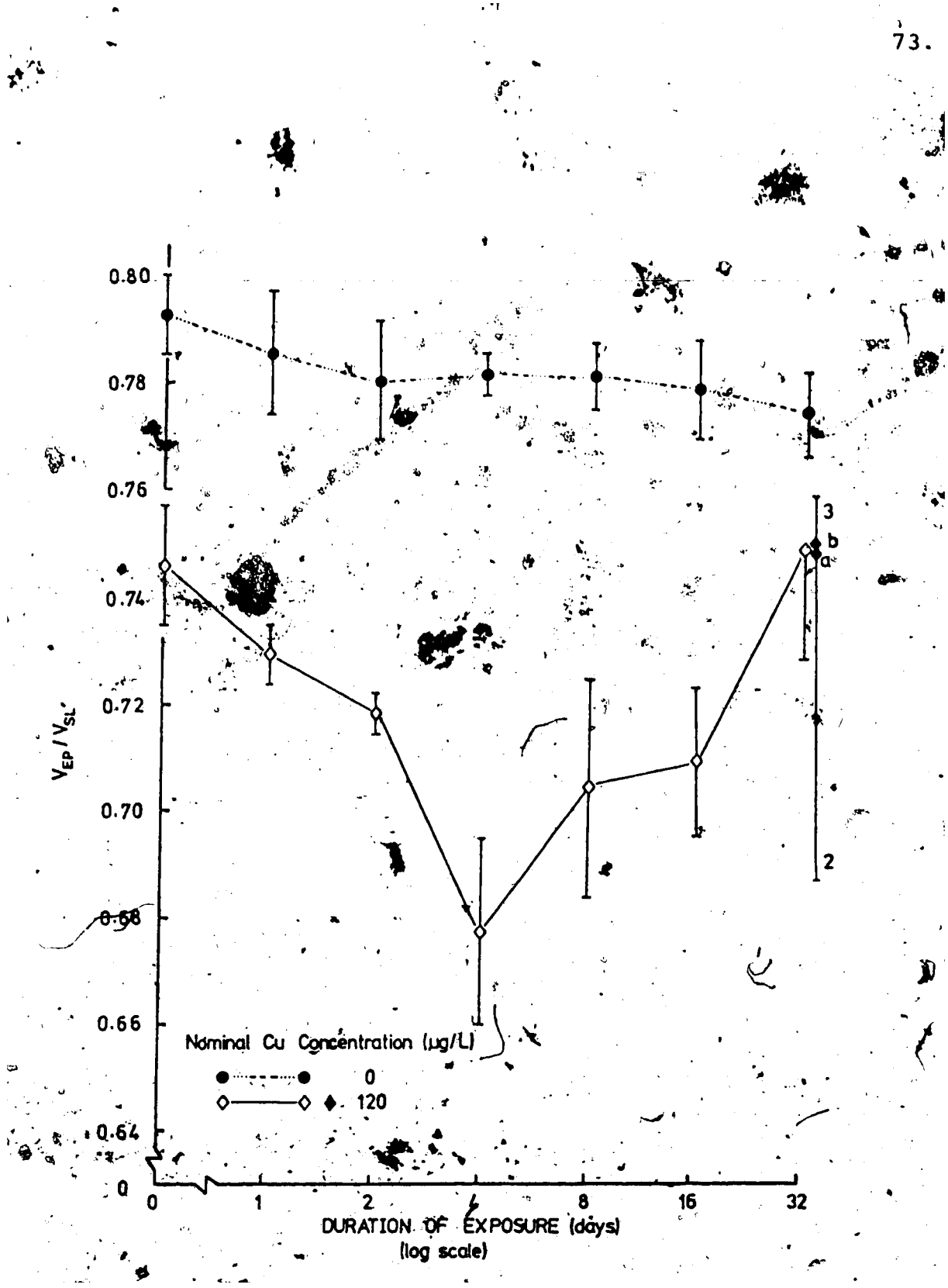

0.80
0.78
0.76
0.74
0.72
0.70
0.68
0.66
0.64
0
V_{EP} / V_{SL}
3
b
a
2
Nominal Cu Concentration (µg/L)
0
120
0
1
2
8
16
32
DURATION OF EXPOSURE (days)
(log scale)

TABLE 10. Correlation coefficients (Spearman's r) between $V_{EP}/V_{SL'}$ and the physiological parameters of trout chronically exposed to copper, series B.

Physiological Parameter	$V_{EP}/V_{SL'}$[1] Exposure		
	0 µg/L Cu	120 µg/L Cu	0 and 120 µg/L Cu combined
Plasma Chloride (mM/L)	NS	NS	** (0.380)[2]
Plasma Sodium (mM/L)	NS	NS	** (0.334)
Plasma Potassium (mM/L)	NS	NS	NS
Plasma Glucose (mg/100 mL)	* (-0.346)	* (-0.365)	** (-0.530)
% Body Water (W/W)	NS	NS	* (0.292)

*$p < 0.05$; **$p < 0.01$,

[1]Volume of the gill epithelia (V_{EP}) relative to the volume of the secondary lamellae including the adjacent epithelia of the primary lamella ($V_{SL'}$),

[2]Spearman's r indicated in brackets where significant.

in the proportion of epithelia preceded the maximum changes in the physiological parameters (Figures 5,6,13). A significant negative correlation was also noted between V_{EP}/V_{SL}, and the plasma levels of glucose (Table 10).

S_I/S_O

A significant difference ($p < 0.01$) was found between the 0 and the 120 µg/L copper-exposed fish for the ratio of the pillar system surface area (S_I) to the epithelial surface area (S_O) in regions A and B of the gill filaments (Table 11). Due to the large fluctuation in values for the 120 µg/L Cu-exposed fish (Figure 16), the biological significance of that difference is questionable. However, if the difference between the results in the control and the 120 µg/L Cu exposures are taken to be biologically significant, it would appear that copper caused the pillar system as a whole to decrease in size (and surface area) relative to the rest of the lamella or that copper caused the loosening of the epithelium from the basement membrane so that the epithelium stretched more in the gills of copper-exposed fish than in the control fish during preparation of the gill sections (see Appendix XII). A change in the proportional volume of the pillar system relative to the secondary lamella (V_{PS}/V_{SL},) could not be reliably determined due to the unexplained differences in the proportion of epithelia

TABLE 11. Results of statistical tests to determine significant differences in S_I/S_O[1] (regions A and B)[2] in trout chronically exposed to copper, series B. Comparisons between concentrations of exposure and between days of exposure.

Statistical Test	Independent Variable: Concentration	Independent Variable: Day
Hodges-Lehmann Conditional Rank Test	**	NS
Groups Significantly[3] Different ($p < 0.05$)	0 + 120	NS

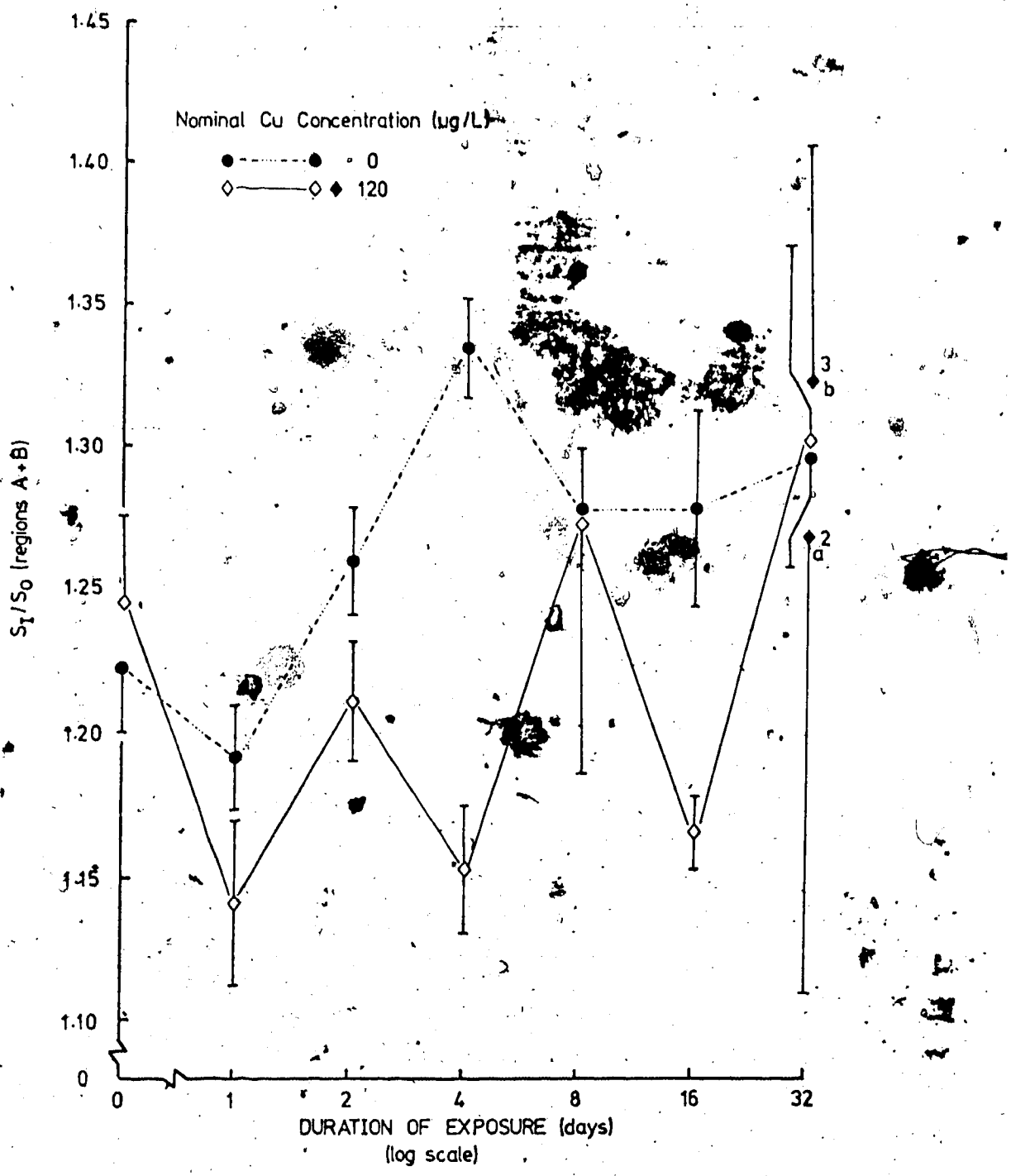
Statistical Test	Nominal Cu Concentration (µg/L): 0	120 with all day 32 results	120 with[4] day 32a results	120 with[4] day 32b results
Kruskal-Wallis Analysis of Variance	*	NS	NS	NS
Days Significantly[3] Different ($p < 0.05$)	NS	NS	NS	NS
Test for Curvilinearity	NS	NS	NS	NS

[1] Surface area of the gill pillar system (S_I) relative to the outer surface area of the gill epithelia (S_O).

[2] Values determined from regions A and B of the gill filament were averaged. Region A = proximal third of gill filaments. Region B = middle third of gill filament.

[3] Pairwise comparisons based on mean ranks, [4] See Figure 16 for definition of day 32a and 32b results.

Figure 16. Ratio between the surface area of the gill pillar system and the outer surface area of the gill epithelium (S_I/S_O) (mean ± S.E.M.) for regions A and B (averaged) of the gill filaments. The ratios were determined from trout chronically exposed to 0 and to 120 µg/L Cu. Region A was the proximal third of the gill filaments. Region B was the middle third of the gill filaments. Except where noted each point represents the mean value of 5 fish. The point indicated at (a) represents the mean value of fish sampled on day 32 which had plasma Cl^- and Na^+ concentrations near the control mean. The point indicated at (b) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ concentrations well below the control mean.


Nominal Cu Concentration (µg/L)
0
120
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
0
S_T/S_0 (regions A+B)
3
b
2
a
0
1
2
4
8
16
32
DURATION OF EXPOSURE (days)
(log scale)

in the day 0 fish (see the section on V_{EP}/V_{SL}).

Significant correlations were found between two parameters (glucose and percent body water) and S_I/S_O from regions A and B (Table 12). Due to the fluctuations in the surface area ratios (Figure 16) and the fact that separation of the epithelia from the pillar systems (Appendix XII) could have altered the surface area of the epithelia, the biological significance of these correlations is questionable.

No significant differences over time were found for the S_I/S_O ratios from region C of the gill filaments. The smaller sample sizes from region C may have contributed to a lack of significance (Figure 17). However, a reduction in the values for the 120 µg/L Cu-exposed fish compared to the control fish appeared to have started between days 8 and 16.

No significant correlations occurred between S_I/S_O (region C) and the physiological parameters.

TABLE 12. Correlation coefficients (Spearman's r) between S_I/S_O (regions A and B) and the physiological parameters of trout chronically exposed to copper, series B.

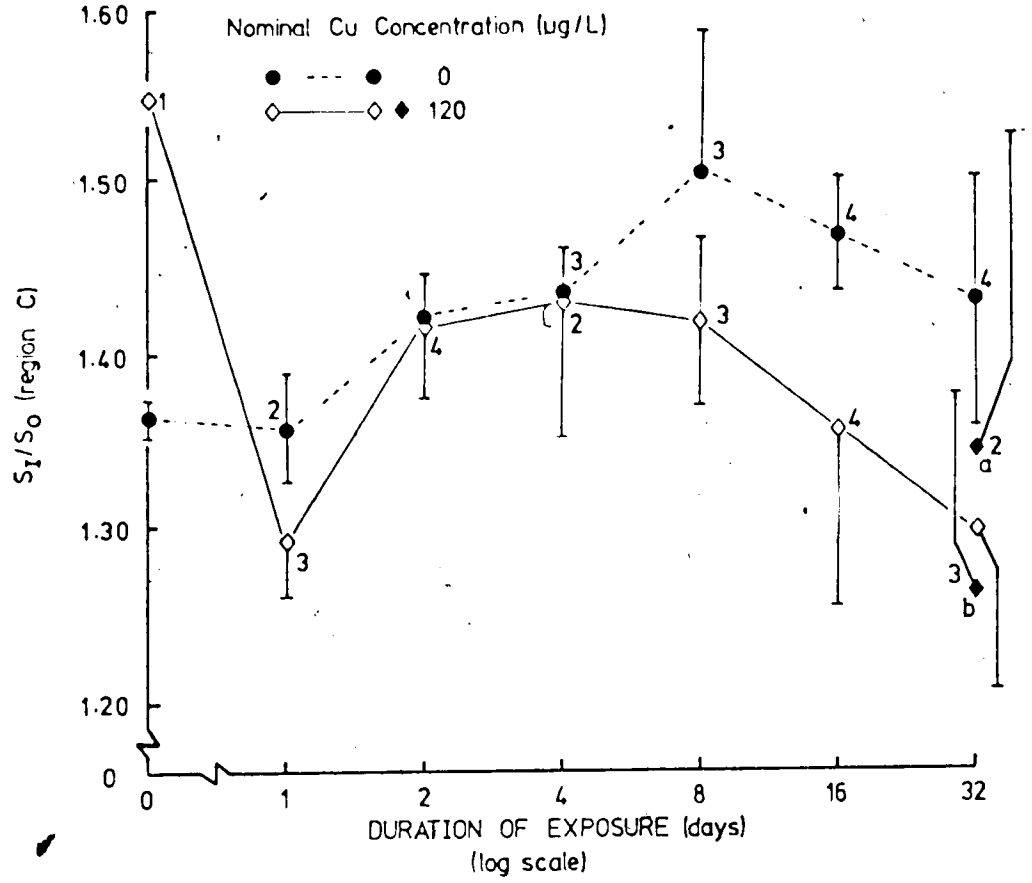
Physiological Parameters	S_I/S_O (regions A+B)[1] Exposure		
	0 µg/L Cu	120 µg/L Cu	0 and 120 µg/L Cu combined
Plasma Chloride (mM/L)	NS	NS	NS
Plasma Sodium (mM/L)	NS	NS	NS
Plasma Potassium (mM/L)	NS	NS	NS
Plasma Glucose (mg/100 mL)	NS	NS	* (-0.259)[2]
% Body Water (W/W)	NS	NS	* (0.260)

*$p < 0.05$, **$p < 0.01$,

[1]See Table 11 for definition,

[2]Spearman's r indicated in brackets where significant.

Figure 17. Ratio between the surface area of the gill pillar system and the outer surface area of the gill epithelium (S_I/S_O) (mean ± S.E.M.) for region C of the gill filament. The ratios were determined from trout chronically exposed to 0 and to 120 µg/L Cu. Region C was the distal third of the gill filament. Except where noted each point represents the mean value of 5 fish. The point indicated at (a) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ concentrations near the control mean. The point indicated at (b) represents the mean value of the fish sampled on day 32 which had plasma Cl^- and Na^+ concentrations well below the control mean.


Nominal Cu Concentration (ug/L)
0
120
1.60
1.50
1.40
1.30
1.20
0
S_I/S_O (region C)
0
1
2
4
8
16
32
DURATION OF EXPOSURE (days)
(log scale)
a
b

DISCUSSION

I. Adaptation to Chronic Copper Exposure

The changes that occurred in the plasma parameters measured appear to confirm that trout can adapt to copper. A decline in the plasma concentrations of Cl^- and Na^+ during the first eight days of exposure was reversed by day 16 during the 120 µg/L Cu exposure (Figures 5,6). A similar decline and recovery was observed for the plasma Cl^- levels of fish exposed to 50 and to 80 µg/L Cu (Figure 5). An increase in plasma glucose levels followed by a return to near control levels was also noted in the fish exposed to 120 µg/L Cu (Figure 8). Similar transient changes in the plasma chloride levels of brown bullheads (*Ictalurus nebulosus* Rafinesque) and brook trout (*Salvelinus fontinalis*) and in the plasma glucose levels of brown bullheads chronically exposed to copper have been reported (McKim *et al.*, 1970; Christensen *et al.*, 1972), though the duration of the transient period had not been determined in those studies.

It may be argued that mortality of fish during exposure to copper, especially during exposure to 120 µg/L Cu, may have been the basis for the changes and apparent recovery. Approximately 25% of the experimental fish died during the exposure to 120 µg/L Cu (which was

approximately 1.2 toxic units, one toxic unit being defined by Sprague (1970) as the LC_{50}), mainly during the first 15 day of exposure (Appendix IX). Thus, the initial changes in the plasma parameters may, in part, have been measured from fish that would not have recovered even though no overt signs of impending death were evident when the fish were sampled. Conversely, the recovery noted from fish sampled at day 16 and from some of the fish sampled at day 32 (Figures 5,6) may have been, in part, the result of sampling fish that did not react significantly to copper. However, such an argument cannot explain the decline and recovery noted in the plasma Cl^- levels of the fish exposed to 50 and to 80 µg/L Cu since only one fish died at day 25 during the 80 µg/L Cu exposure and only three died during the 50 µg/L Cu exposure between days 57 and 62 (Appendix IX).

The continued decline in the plasma concentrations of Cl^- and Na^+ in some of the trout sampled on day 32 of the 120 µg/L Cu exposure (Figures 5,6) and the re-occurrance of an elevated plasma glucose level in those same fish (Figure 8) may represent the exhaustion stage of the General Adaptation Syndrome (G.A.S.) as described by Selye (1950). The initial changes in the plasma parameters would represent the "alarm reaction" of the G.A.S. while the recovery would represent the "resistance stage" of the G.A.S. Whether all the fish

remaining within the resistance stage after 64 days of exposure would have eventually reached an exhaustion stage is not known. The survivors among brook trout exposed for 11 months and of brown bullheads exposed for 600 days to copper concentrations that had caused significant decreases in plasma ion levels did not have plasma ion levels significantly different from the levels in the control fish (McKim *et al.*, 1970; Christensen *et al.*, 1972). This would suggest that some of the fish did not reach an exhaustion stage (i.e., that they adapted).

The plasma levels of K^+ were found to decline at the 100 and 120 µg/L Cu exposures (Figure 7). The increase in plasma K^+ levels to near control levels in some of the fish sampled on day 32 (day 32a sample) of the 120 µg/L Cu exposure may be indicative of a recovery since the K^+ value from fish sampled at day 16 was significantly lower than the K^+ value measured from the control fish on day 16. No previous studies have reported changes in plasma K^+ with exposure of fish to heavy metal pollution.

II. Relationship Between Stress and Hydromineral Changes Due to Copper.

A stress response in trout exposed to copper was shown by the occurrence of elevated levels of plasma glucose (Figure 8). The existence of a stress response in fish during exposure to copper has been confirmed by

two other studies (Donaldson and Dye, 1975; Schreck and Lorz, 1978) in which elevated corticosteroid levels were measured in salmon (*Oncorhynchus nerka* and *O. kisutch*) exposed to copper.

The stress response in trout chronically exposed to copper appears to be most closely related to a reduction in the plasma concentrations of Cl^- and Na^+. This was indicated by highly significant ($p = 0.001$) negative correlations between the plasma glucose levels and the plasma levels of Cl^- and Na^+ (Figures 9,10). Increased plasma glucose concentration, considered a secondary effect of increased stress (Mazeaud *et al.*, 1977), is probably caused by elevated circulatory levels of corticosteroids and catecholamines (Frye, 1967). Since glucose levels probably do not influence ion levels directly, the possible means by which corticosteroids and catecholamines influence ion levels is discussed below.

Cortisol, a corticosteroid, may be involved in correcting a loss of ions in freshwater fish. Cortisol apparently increased the reabsorption of Na^+ from the kidneys of fish (de Vlaming, 1979) and injections of physiological doses of cortisol into freshwater-adapted eels (*Anguilla anguilla*) promoted uptake of Na^+ across the gills (Chan *et al.*, 1969). Facilitation of Na^+ influx by cortisol in freshwater-adapted euryhaline fish has also been

reported by several authors (review by Johnson, 1973). Though many researchers (review by: Butler, 1973; Johnson, 1973; Henderson and Chester Jones, 1974) have associated cortisol primarily with the adaptation of euryhaline fish to seawater and with Na^+ efflux rather than Na^+ influx, cortisol may also have a role in freshwater-adapted fish through its influence on Na^+/K^+ ATPase activity. Cortisol increased Na^+/K^+ ATPase activity in intact and hypophysectomized freshwater-adapted eels (*A. rostrata*) (Butler, 1973; Epstein *et al.*, 1971) which may aid Na^+ influx. Some studies, however, have shown that cortisol inhibited rather than enhanced Na^+ uptake across the gills of freshwater-adapted fish (review by Chester Jones *et al.*, 1969) but that inhibition may have been due to the use of pharmacological doses of cortisol rather than physiological doses (Butler, 1973).

Thus, elevation of cortisol levels in fish exposed to copper may represent a physiological attempt to correct a loss of Na^+. Since copper has been shown to inhibit Na^+/K^+ ATPase activity *invitro* (Hexum, 1974; Ting-Beall *et al.*, 1973; Bowler and Duncan, 1970) and *in vivo* (Lorz and MacPherson, 1976), the extent of elevation of Na^+/K^+ ATPase activity by cortisol may be dependent on the degree of Na^+/K^+ ATPase inhibition caused by copper.

Adrenalin, a catecholamine, may also counterbalance a decrease in sodium ions that occurs with copper exposure

(Figure 6 and Courtois, 1976) by increasing Na^+ uptake in the gills (Richards and Fromm, 1970; Payan *et al.*, 1975). Recently Payan and Girard (1978) have shown that adrenalin stimulated the Na^+/NH_4^+ exchange in the secondary lamellae of trout. Furthermore, adrenalin appears to increase gill permeability to oxygen (Payan *et al.*, cited in Girard and Payan, 1980) and earlier work by Randall *et al.* (1972) had shown a probable link between oxygen transfer rate and ion fluxes across the gills of *S. gairdneri*. Since a decrease in oxygen consumption is an effect of copper exposure on fish (O'Hara, 1971), adrenalin may help to counterbalance or limit the reduction in oxygen consumption which, in turn, would help limit copper-induced ion losses.

The role of the corticosteroids and the catecholamines in maintaining the hydromineral balance seems to be primarily related to the control of Na^+ in fish (see above); but, in the present study, both the plasma Na^+ and Cl^- levels were correlated with the stress (glucose) levels (Figures 9,10). The correlation between plasma glucose and plasma Cl^- may be explained by recent studies which have indicated that the uptake mechanisms for Na^+ and Cl^- are interrelated (review by Maetz, 1974), apparently through the regulation of acid-base balance and ammonia clearance (review by Girard and Payan, 1980).

Though no significant changes over time occurred in the total body water of trout exposed to copper, significant negative correlations ($p < 0.05$) were noted between percent body water and the plasma levels of Cl^- and Na^+ of the trout exposed to 120 µg/L Cu (Figures 11,12), indicating that some of the reduction in ion levels may be due to dilution. Both adrenalin and cortisol have been associated with stress-induced imbibition of water in freshwater fish (Mazeaud *et al.*, 1977). Adrenalin has been shown to increase gill permeability to water in the freshwater-adapted grey mullet (Pic *et al.*, 1974) while cortisol has increased the osmotic water influx into isolated gills of freshwater-adapted Japanese eels (*Anguilla japanica*) (Ogawa, 1975). However, cortisol has also been shown to restore a diminished urine output in freshwater-adapted eels after adrenalectomy (Chan *et al.*, 1969), so some of the stress-induced dilution may be counteracted.

III. Relationship Between Gill Structure and Hydromineral Changes Due to Copper.

Separation of the epithelia from the basement membrane occurred in the gill sections from both the control and the copper-exposed fish (Appendix XII). Such an artefact likely introduced a systematic error in the measurement of some of the gill parameters. With separation, the epithelia likely stretched. Stretching would be expected

to significantly change the estimation of the outer surface area of the epithelia (S_O). The erratic fluctuations seen in the measurements of the S_I/S_O ratio (Figure 16) suggest that the stretching was not uniform across samples. Thus, any correlations that occurred between S_I/S_O and the plasma parameters will not be further discussed.

The possible stretching of the epithelia may have also introduced a systematic error in the estimation of V_{EP}/V_{SL}, but, it appears that the error was uniform across samples since very little change occurred over time in the control fish. Thus, the relationships noted with V_{EP}/V_{SL}, may still have been representative of actual relationships in the living animal. It is unlikely that the V_{BC}/V_{PS} ratios were affected by separation of the epithelia since the pillar systems remained intact.

V_{BC}/V_{PS}

Only the plasma potassium level was significantly correlated with the relative volume of the blood channel spaces in the pillar system (Table 8). Dilation of blood vessels in teleost gills has been associated with increases in heart rate and blood pressure (Smart, 1976) and with an increase in blood flow in the secondary lamellae (Steen and Kruysse, 1964). With an increase in blood flow through the gills, the potential for greater diffusion or exchange of substances would exist. However, an

increase in blood flow through the gills was likely a minor factor in the diffusion or exchange of potassium since no correlations were observed between V_{BC}/V_{PS} and the plasma levels of Na^+ and Cl^- - ions present in much greater concentration in the blood than K^+ (Holmes and Donaldson, 1969).

V_{EP}/V_{SL}'

No correlations were observed between the relative volume of the epithelia in the gill and the ion or the water concentrations in the fish when the two treatment groups (0 and 120 µg/L Cu) were considered separately (Table 10). However, when the data of the two groups were analyzed together, significant correlations were observed between V_{EP}/V_{SL}' and plasma Cl^-, plasma Na^+ and percent body water (Table 10), indicating that changes in the proportion of gill epithelium, regardless of cause, influences ion and water levels in trout.

In the present study a positive correlation occurred between V_{EP}/V_{SL}' and the plasma levels of Cl^- and Na^+ (Table 10), indicating that a reduction in gill epithelium was associated with a reduction in the plasma levels of Na^+ and Cl^- (see Figures 5,6,15). This association appears to be true for trout exposed to cadmium, another heavy metal. McCarty and Houston (1976) reported that chronic exposure of trout to cadmium reduced the plasma

levels of Na^+ and Cl^-. Hughes *et al.* (1979) noted a decrease in the proportion of epithelia in the secondary lamellae of trout exposed to cadmium (data recalculated from the tables reported*). The decrease in the proportion of epithelia was not significant but the concentrations of cadmium used by Hughes *et al.* (2-8 µg/L) were much lower than the concentrations used by McCarty and Houston (44.5 and 380 µg/L).

In contrast to the reduction in gill epithelium during exposure to copper noted in the present study (Figure 15), Schreck and Lorz (1978) reported hyperplasia in the gills of freshwater-adapted coho salmon (*O. kisutch*) exposed to sublethal concentrations of copper. Systematic error in estimating epithelial volumes may have occurred in the present study due to separation of the epithelia from the basement membrane. However, a reduction in the thickness of the interlamellar epithelium was observed

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*The data reported by Hughes *et al.* (1979) was recalculated as follows to yield a new value (V_{tis}/V_{SL}):

$$V_{OPS}/V_{SL} \times V_{tis}/V_{OPS} = V_{tis}/V_{SL}$$

V = volume
OPS = region exterior to the basement membrane in the secondary lamella
SL = secondary lamella
tis = tissue occupied regions of the OPS. This did not include the interlamellar (primary lamellar) epithelium.

in *Fundulus heteroclitus* exposed to copper in seawater (Baker, 1969). Whether exposure of fish to copper in seawater or in freshwater would make a difference on the effects of copper on gill structure remains to be studied. Also, further research would be required to determine whether the contrasting observations in the present study and the study by Schreck and Lorz (1978) are due to species differences in the response to copper or due to a systematic error that may have occurred in the present study.

The relationship between percent body water and V_{EP}/V_{SL}, (Table 10) appears to be an anomoly. A positive correlation was observed between those two parameters but negative correlations were observed between percent body water and the plasma levels of Na^+ and Cl^- (Figures 11,12) and between V_{EP}/V_{SL}, and the plasma levels of Na^+ and Cl^- (Table 10). Since an increase in water diffusion is more likely with a decrease in the barrier between the external and the internal environments of the fish, the positive correlation between V_{EP}/V_{SL}, and percent body water was likely a type II error as described by Sokal and Rohlf (1969), possibly due to a systematic error.

A significant negative correlation ($p < 0.01$) occurred between V_{EP}/V_{SL}, and plasma glucose (Table 10), indicating that a decrease in epithelial volume was associated

with an increase in stress. Since biochemical changes generally precede morphological changes (Anderson, 1961) and since little is known about the effects of stress-related hormones on gill structure, the biological significance of that correlation can only be guessed.

IV. Conclusions

Trout exposed to 50, 80 and 120 µg/L Cu appear to exhibit a General Adaptation Syndrome as defined by Selye (1950), at least with respect to the plasma concentrations of Cl^-, Na^+ and glucose. The first two stages, the alarm reaction and the resistance stage, were evident in the fish exposed to all three concentrations of copper. Some of the fish exposed to 120 µg/L Cu also exhibited the exhaustion stage.

The highly significant correlations between plasma glucose and the plasma levels of Cl^- and Na^+ suggests that the ability of trout to adapt to copper with respect to hydromineral balance may be dependent on the ability of the stress response to counterbalance ionic changes induced by copper. It is suggested that elevated levels of catecholamines and corticosteroids, which are part of a stress response (Mazeaud *et al.*, 1977), may play a role in counterbalancing copper-induced changes.

The role of structural changes in the gill on hydromineral balance is unclear. It appears that a

reduction in the plasma levels of Cl^- and Na^+ and an increase in plasma glucose levels may be associated with a reduction in the proportion of epithelia in the gill, but other studies (e.g. Schreck and Lorz, 1976) have reported hyperplasia of the gill with exposure of fish to copper. A reduction in plasma potassium levels appears to be associated with an increase in the relative volume of the blood channel spaces in the pillar system, but no explanation for this association can be given.

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APPENDICES

Appendix I. Water quality parameter measurements from individual test tanks. The range and number of samples (N) are listed when more than one sample was measured for a given parameter.

A. Water quality during the determination of the LC_{50} of copper to rainbow trout.

	Nominal Cu Concentration (µg/L)	pH	Conductance (µmhos)	Acidity as $CaCO_3$ (mg/L)	Total Alkalinity as $CaCO_3$ (mg/L)	Calcium Hardness as $CaCO_3$ (mg/L)	Total Hardness as $CaCO_3$ (mg/L)	Chloride (mg/L)	Sodium (mg/L)	Potassium (mg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	Total Copper (µg/L)	Dissolved Copper (µg/L)	95% Replacement Time of Water (h)
TEST A	0	7.42	268		22.0	121	129	85.1	8.6	1.5	9.3-9.5 (2)		1.35[a]	3.25	29.5
	70	7.46	262		21.2	127	136	82.9	7.6	1.4	9.8-9.9 (2)		100[a]	51.6	29.0
	100	7.54	273		20.0	121	136	85.1	8.8	1.5	9.9-10.1 (2)		107.5[a]	105	27.5
	130	7.43	281		1.0	123	136	87.6	8.8	1.5	9.7		130[a]	127.5	28.0
TEST B	0	7.18-7.41 (2)									9.7-11.9 (7)		0-<3[a] (3)		20-26.2 (3)
	75	6.87-7.36 (2)									9.5-11.8 (7)		73-73.5[a] (2)		21-27 (3)
	85	6.95-7.27 (2)									9.4-11.3 (7)		78.5-82[a] (2)		20.5-27 (3)
	95	7.12-7.39 (2)									9.7-10.9 (7)		61-87[a] (5)		20-26 (3)
TEST C	0	6.95	327	5.9	2.0	122	128	87.5	7.4	1.6	10.5-11.1 (10)	9.00-9.38 (3)	<1-1.5[a] (3)		
	120	6.69	267	11.1	23.0	132	136	83.3	6.8	1.6	10.1-10.8 (10)	9.38-9.49 (3)	96-125[a] (4)	90-115 (4)	
													116-127[b] (3)		
	140										10.2-10.9 (10)	9.21	73.5-113[a] (3)	104-111 (3)	
													136-139[b] (2)		
	160										10.5-10.8 (6)	9.28	140-150[a] (2)	118-135 (2)	
													155-162[b] (2)		

[a] Calculated according to Sprague (1969),

[a] Total copper as measured by method A - acidification of sample with no filtration (Appendix V),

[b] Total copper as measured by method B - filtration of sample through a 0.45 µm membrane filter after acidification (Appendix V).

B. Water quality during chronic exposure of rainbow trout to copper.

	Nominal Cu Concentration (µg/L)	pH	Conductance (µmhos)	Acidity as $CaCO_3$ (mg/L)	Total Alkalinity as $CaCO_3$ (mg/L)	Calcium Hardness as $CaCO_3$ (mg/L)	Total Hardness as $CaCO_3$ (mg/L)	Chloride (mg/L)	Sodium (mg/L)	Potassium (mg/L)	Temperature (°C)	Dissolved Oxygen (mg/L)	Total[a] Copper (µg/L)	Dissolved Copper (µg/L)	95%[a] Replacement Time of Water (h)
SERIES A	0	7.23-7.28 (2)	221-322 (4)	5.0-10.5 (4)	19.5-27.3 (4)	104-132 (4)	108-140 (4)	72.9-100.3 (4)	7.0-14.5 (4)	0.0-2.4 (4)	9.3-10.8 (17)	-	0.0-2.05 (9)	-	20-21 (4)
	20	7.45	288-297 (2)	6.8-8.0 (2)	12.8-22.0 (2)	128-131 (2)	128-140 (2)	91.2-92.8 (2)	8.0-9.0 (2)	2.0-2.8 (2)	9.5-11.3 (13)	-	17.5-22.5 (9)	14-14.5 (2)	22-26 (4)
	50	7.51	210-268 (2)	2.5-4.0 (2)	16.4-21.0 (2)	103-122 (2)	124 (2)	73.7-86.2 (2)	5.0-8.0 (2)	0.0-1.5 (2)	9.8-10.9 (13)	-	36-47 (9)	32-35 (2)	21-23 (4)
	80	7.39	258-310 (2)	5.0-11.0 (2)	22.8-25.8 (2)	124-132 (2)	144-148 (2)	86.8-93.9 (2)	8.0-8.8 (2)	1.2-2.4 (2)	9.2-11.2 (15)	-	29.5-66 (9)	40-54 (2)	21.5-26 (4)
SERIES B	0	7.10	-	4.0	23.0	118	120	85.2	7.3	2.4	10.2-12.1 (23)	8.72-9.00 (3)	0-6.8 (9)	-	21-34 (3)
	50	7.13-7.18 (2)	265	11.5	20.4	122	128	84.5	8.8	2.0	9.6-12.1 (17)	8.49-8.93 (2)	32.8-51.0 (9)	17.5-50 (9)	20-52 (5)
	80	7.15-7.18 (2)	270-280 (2)	2.9-10.0 (2)	22.0-23.0 (2)	115-116 (2)	120-136 (2)	82.8-84.2 (2)	7.2-8.0 (2)	1.6-2.0 (2)	10.5-12.5 (19)	8.65-8.74 (2)	45-81 (9)	29.5-59 (9)	20.5-25 (6)
	100	7.16	279	12.0	22.2	140	142	85.7	17.5	1.6	10.5-12.3 (4)	-	52.5-94 (6)	20.2-43.5 (6)	20-42 (3)
	120	7.13	240-283 (3)	3.0-5.0 (3)	19.5-24.2 (3)	118-120 (3)	128-136 (3)	84.2-85.6 (3)	5.0-10.0 (3)	1.4-2.7 (3)	10.2-12.0 (21)	8.67-8.88 (3)	53-119 (8)	38.5-92 (8)	20-21.5 (3)

[a] Total copper as measured by method A - acidification of sample with no filtration (Appendix V).

[a] Calculated according to Sprague (1969).

Appendix II. Body weight and length of fish used during the determination of the LC_{50} of copper to trout.

Test	Nominal Total Cu Concentration (µg/L)	No. Fish (N)	Wet Weight (g)	Standard Length (cm)
A	0	12	11.5-40.8[1] 25.5±7.9[2]	9.0-13.7 11.9±1.3
	70	12	18.6-34.9 24.1±4.8	11.3-13.5 12.1±0.7
	100	12	18.6-38.5 26.7±7.4	10.7-14.6 12.2±1.1
	130	12	20.2-32.7 25.8±4.4	11.1-13.3 12.1±0.7
	TOTAL	48	11.5-40.8 25.5±6.2	9.0-14.6 12.1±1.0
B	0	10	13.9-42.7 28.8±8.9	11.6-15.4* 13.9±1.2
	75	10	16.9-27.9 22.5±4.4	12.1-14.2* 13.1±0.8
	85	10	14.8-34.4 23.5±5.8	11.2-14.4* 13.3±1.0
	95	10	18.3-28.8 23.2±4.2	12.3-14.9* 13.5±0.9
	TOTAL	40	13.9-42.7 24.6±6.5	11.2-15.4* 13.5±1.0
C	0	12	26.7-52.9 41.1±8.1	12.6 15.7 14.5±0.9
	120	12	26.0-40.5 35.0±4.2	12.4-14.8 13.8±0.8
	140	12	23.7-47.3 34.2±7.7	12.7-15.6 13.9±1.0
	160	12	25.5-50.5 37.6±7.2	12.8-16.0 14.1±1.0
	TOTAL	48	23.7-52.9 36.2±8.8	11.2-16.0 14.1±0.9

[1]Range, [2]Mean ± S.D., *Fork length.

Appendix III. Determination of the time required for the nominal Cu concentration to be achieved in a test tank. The water samples were taken from the 95 µg/L Cu test tank during Test B of the LC_{50} determinations.

Time After Start of Cu Addition (h)	Total [Cu]* (µg/L)
2	28.5
7	64
14	67.5
24	61
49.5	87
240	84

*Measured by method A - acidification of the sample with no filtration. The values reported here were likely lower than the actual copper concentration in the tank due to artefacts in the analysis method used (see Appendix V).

Appendix IV. Body weight and length of fish used in the chronic exposure tests.

Series	Nominal Total Cu Concentration (µg/L)	No. fish (N)	Wet Weight (g)	Wet Weight Without Gills (g)	Dry Weight (g)	Standard Length (cm)
A	0	43	31.7-70.7[1] 49.1±10.2[2]			13.4-18.0 15.6±1.0
	20	46	11.5-49.7 24.2±7.7			10.7-15.0 12.5±1.0
	50	46	14.4-50.0 28.6±7.7			10.7-15.5 12.9±1.0
	80	44	22.8-65.6 44.8±11.1			12.0-16.9 15.1±1.4
B	0	50	18.9-64.1 30.0±9.5	16.5-59.5 27.9±9.0	3.28-14.69 6.51±2.34	10.8-15.9 12.8±1.2
	50	52	26.4-69.2 41.4±10.1	25.2-66.2 39.2±9.6	5.50-14.40 9.42±2.45	12.9-17.0 14.7±1.1
	80	51	13.1-68.3 30.9±10.8	11.7-63.4 29.5±9.4	2.23-16.53 6.82±2.53	10.8-15.8 13.3±1.3
	100	31	25.9-53.1 37.0±7.1	23.3-50.5 35.9±6.8	5.40-11.03 8.08±1.51	12.3-16.2 14.3±1.0
	120	49	19.9-44.8 30.8±6.5	18.6-42.3 29.1±6.2	4.02-9.77 6.82±1.53	11.0-14.6 13.2±1.0

[1]Range, [2]Mean ± S.D.

Appendix V. The effect of different methods of sample collection on copper analysis.

During the course of the chronic exposure experiment, it was noticed that the measured value for dissolved copper was sometimes higher than the measured value for total copper.

According to Traversy (1971) an unfiltered, water sample that has been acidified yields total copper if all the sediment was dissolved. If some sediment remained undissolved, some of the copper may bind to the sediment so strongly, that it could not be extracted by the APDC-MIBK extraction procedure used to measure copper in the present study. If such bound copper could not be extracted, then the measured total copper would be below the true concentration.

In order to determine if the presence of sediment during storage of copper samples could influence the measured values of copper, several water samples for copper analysis were taken during the course of Test C of the LC_{50} determinations and treated in one of the following ways before storage:

(i) no filtration - should yield total copper (= Method A)

(ii) filtration through a 0.45 µm membrane filter after acidification - should yield total copper (= Method B)

(iii) filtration through a 0.45 µm membrane filter before acidification - should yield dissolved copper (Traversy, 1971).

The samples were analyzed by atomic absorption spectrophotometry as described in Material and Methods.

As shown in Figure V-1, the total copper values measured by Method B yielded the values closest to the nominal copper concentrations. In most cases, the dissolved copper samples yielded the lowest measured concentration, as expected. However, in some instances (e.g., samples A' and B'), the total copper concentration as measured by Method A yielded values lower than the dissolved copper values. This latter result, and the fact that total copper concentrations measured by Method A consistently yielded lower values than those analyzed by Method B, indicates that the presence of sediment in the stored sample results in further binding of copper which could not be later freed by an APDC-MIBK extraction. Filtration of the water sample after acidification appears to prevent futher binding of copper during storage if sediment is present.

Thus, the method used for preparation of the total copper samples (Method A) during most of the experiments in the present study was unreliable.

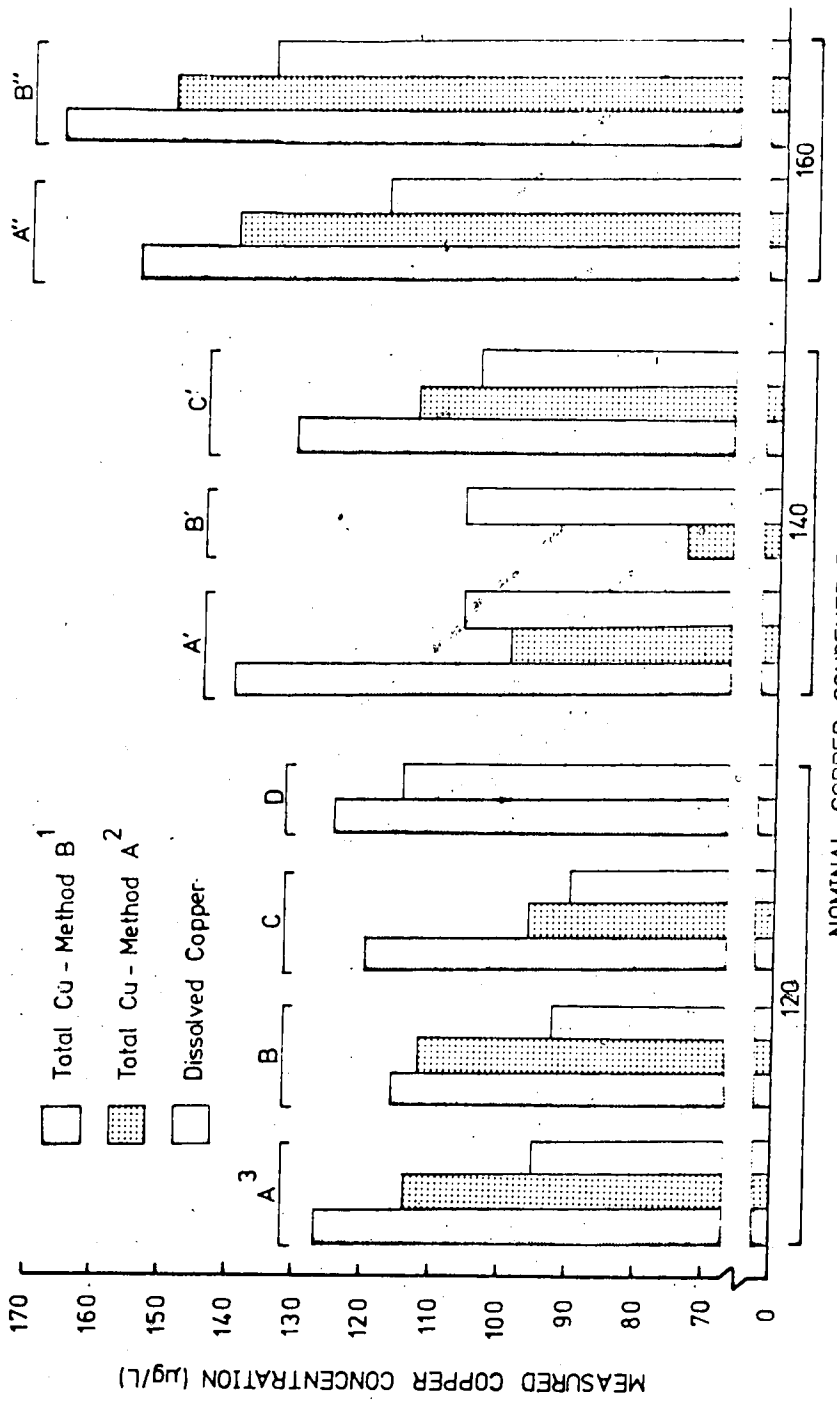


Figure V-1. The effect of different methods of sample collection on measured copper concentration. The water samples were taken from the test tanks during the determination of the LC_{50} of copper to trout (Test C).

[1]The water samples were acidified and then filtered through a 0.45 µm membrane filter,

[2]The water samples were acidified only,

[3]The copper concentrations of water samples taken at the same time are grouped together.

Appendix VI. Water quality analysis methods.

Parameter	Method	Reference
conductance (μmhos)	conductivity bridge (YSI, model 31)	
acidity as $CaCO_3$ (mg/L)	phenolphthalein acidity	A.P.H.A.[1], p.50
total alkalinity as $CaCO_3$ (mg/L)	titration, using phenolpthalein for the first end-point indicator and bromcresol green-methyl red for the second end-point indicator	A.P.H.A., p.52-56
calcium hardness as $CaCO_3$ (mg/L)	E.D.T.A. titritmetric method	A.P.H.A., p.179-184
total hardness as $CaCO_3$ (mg/L)	E.D.T.A. titrimetric method	A.P.H.A., p.179-184
chloride (mg/L)	argentometric method	A.P.H.A., p.96-97
potassium (mg/L)	atomic absorption (Jarrell-Ash Atomic Absorption/Flame Emission Spectrophotometer, Model 82-270)	A.P.H.A., p.318-319; Atomic Absorption Analtyical Method No. 25[2]
sodium (mg/L)	atomic absorption	A.P.H.A., p.284; Atomic Absorption Analytical Method No. 32[2]
dissolved oxygen (mg/L)	azide modification	A.P.H.A., p.477
pH	pH/ion meter (Fisher Accumet, Model 520)	
temperature (°C)	alcohol thermometer	

[1]Taras, M.J., A.E. Greenberg, R.D. Hoak and M.C. Rand (eds), 1971, *Standard Methods for the Examination of Water and Wastewater*, 13th edition. American Public Health Association Washington, D.C.

[2]Anon. 1972. Atomic absorption and analytical methods. Fisher Scientific Company, Jarrell Ash Division, Waltham, Mass.

Appendix VII. Histological techniques.

A. Embedding in paraffin

The gill tissues to be embedded were dehydrated and infiltrated with Paraplast in an automatic tissue processor (Fisher Dual Unit Tissuematon, Model 61) using the following schedule:

1. 90% ethyl alcohol - 1 h
2. Absolute ethyl alcohol I - 1 h
3. Absolute ethyl alcohol II - 1 h
4. Absolute ethyl alcohol:benzene (1:1) - 1 h
5. Benzene I - 0.5 h
6. Benzene II - 0.5 h
7. Paraplast I - 1 h
8. Paraplast II - 1 h

The tissues were then placed in a vacuum infiltrator, which was filled with Paraplast, for 30 minutes and then blocked in molds.

B. Staining

Before staining, the mounted tissue sections were deparaffinized and hydrated as follows:

1. Zylene I - 3 min
2. Zylene II - 3 min
3. Absolute ethyl alcohol - 3 min
4. 90% ethyl alcohol - 3 min

5. 70% ethyl alcohol - 3 min

6. 50% ethyl alcohol - 3 min

7. Distilled water - 4 min

The slides were then stained with Herovici's Polychrome (Herovici, 1963) and dehydrated as follows:

1. 90% ethyl alcohol - dip in and out

2. Absolute ethyl alcohol I - 2 min

3. Absolute ethyl alcohol II - 2 min

4. Zylene I - 3 min

5. Zylene II - 3 min

Cover slips were then placed over the sections using DPX mountant (BDH Chemical, Winnipeg).

Appendix VIII. Median lethal concentration (LC_{50}) of copper to rainbow trout.

A. Table of results

Test A - 4-day LC_{50}[1]

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L) Total Cu (method A)[2]	Measured Dose (µg/L) Dissolved Cu	Percent Mortality 96 h
0	12	1.4	3.2	0
70	12	100	52	8.3
100	12	118	105	91.7
130	12	130	127.5	100
Chi^2		0.038, p>0.05	0.304, p>0.05	
Slope		1.11 (*[a])[3]	1.22 (*[a])	
LC_{50}		109 (*[a])	79 (*[a])	

Test A - 8-day LC_{50}

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L) Total Cu (method A)	Measured Dose (µg/L) Dissolved Cu	Percent Mortality 192 h
0	12	1.4	3.2	0
70	12	100	52	8.3
100	12	118	105	91.7
130	12	130	127.5	100
Chi^2		0.038, p>0.05	0.304, p>0.05	
Slope		1.11 (*[a])	1.22 (*[a])	
LC_{50}		109 (*[a])	79 (*[a])	

Test B - 4-day LC_{50} [1]

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L) Total Cu (method A) [2]	Percent Mortality 96 h
0	10	1.4(0-3) [4]	0
75	9	73.2(73-73.5)	11.1
85	10	80.2(78.5-82.0)	50.0
95	9	85.1(83.5-86.8)	11.1
Chi^2		>38, $p<0.05$	
Slope		1.016(*[b]) [3]	
LC_{50}		80(*[b])	

Test B - 8-day LC_{50}

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L) Total Cu (method A)	Percent Mortality 192 h
0	10	1.4(0-3)	0
75	9	73.2(73-73.5)	11.1
85	10	80.2(78.5-82.0)	60.0
95	9	85.1(83.5-86.8)	11.1
Chi^2		>38, $p<0.05$	
Slope		1.006(*[b])	
LC_{50}		80(*[b])	

Test C - 4-day LC_{50} [1]

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L)			Percent Mortality 96 h
		Total Cu (Method B) [2]	Total Cu (Method A)	Dissolved Cu	
0	12	-	1.2(1-1.5) [4]	-	0
120	12	121(116-127)	112(96-125)	98(90-115)	8.3
140	12	137.5(136-139)	95(73.5-113)	107(104-111)	16.7
160	12	158.5(155-162)	145(140-150)	126.5(118-135)	75.0
Chi^2		0.87,p>0.05	*[c]	0.33,p>0.05	
Slope		1.12(1.04-1.20) [3]	*[c]	1.13(1.04-1.23)	
LC_{50}		148(139-158)	*[c]	118(110-126)	

Nominal Cu Concentration (µg/L)	No. Fish (N)	Measured Dose (µg/L)			Percent Mortality 192 h
		Total Cu (Method B	Total Cu (Method A)	Dissolved Cu	
0	12	-	1.2(1-1.5)	-	0
120	12	121(116-127)	112(96-125)	98(90-115)	83.3
140	12	137.5(136-139)	95(73.5-113)	107(104-111)	91.7
160	12	158.5(155-162)	145(140-150)	126.5(118-135)	100
Chi^2		0.070,p>0.05	*[c]	0.122,p>0.05	
Slope		1.21(0.9-1.6)	*[c]	1.15(0.98-1.36)	
LC_{50}		103(88-120)	*[c]	86.5(77.3-96.8)	

Footnotes for Table of Results

[1]Calculated according to Litchfield and Wilcoxin (1949).

[2]Method of analysis of total copper - see Appendix V. Method A considered unreliable.

[3]95% confidence limits in brackets for slope and LC_{50}.

[4]Range of measured Cu values in brackets when more than one measurement taken.

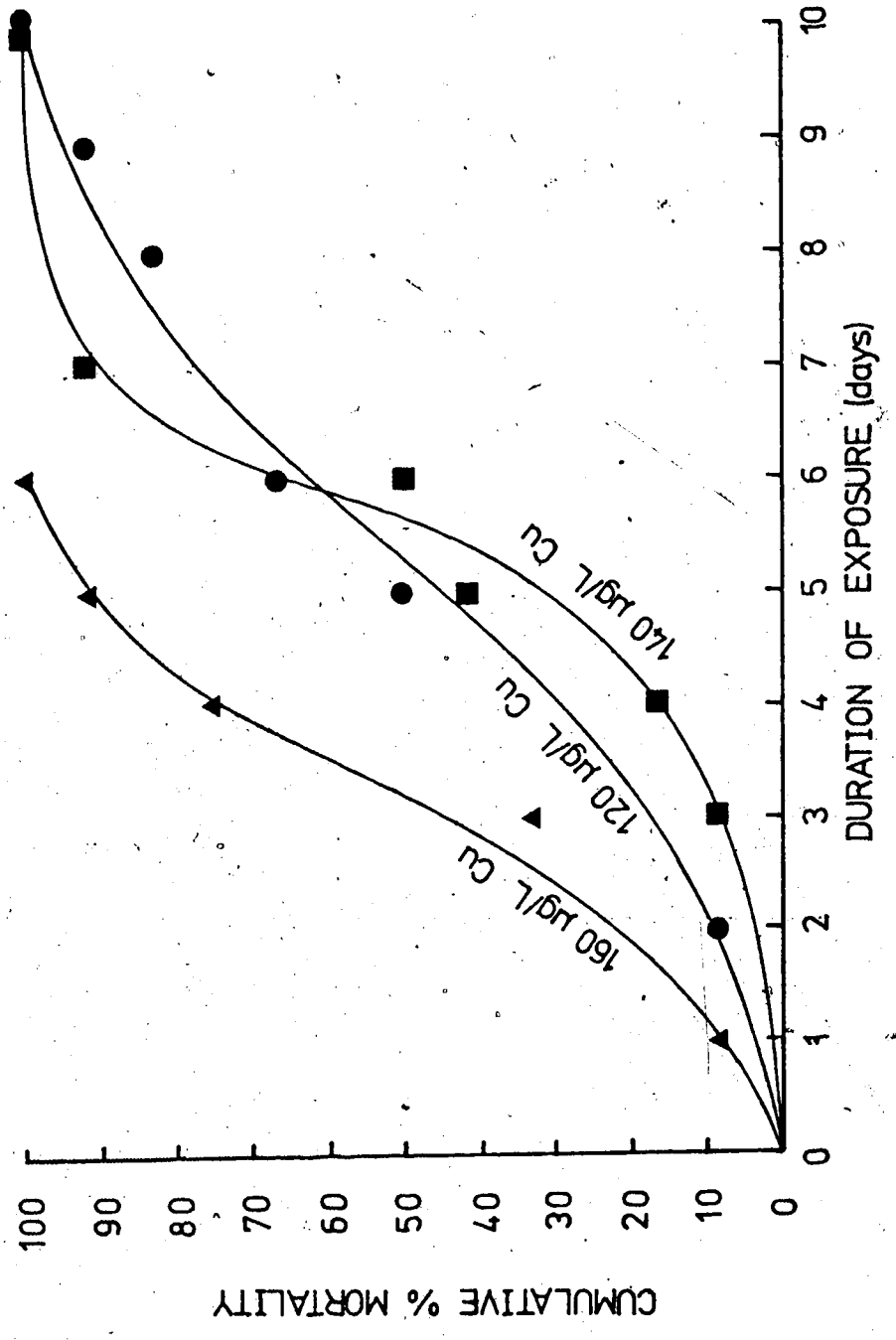
*Unable to calculate, reasons as follows:

a - N' = 0, cannot calculate F_S or F_{ED50} (Litchfield and Wilcoxin, 1949)

b - Cannot determine exact expected percent mortality for at least one dose from log-probability plot, therefore cannot calculate exact Chi^2 needed for F_S or F_{ED50} determination (Litchfield and Wilcoxin 1949)

c - Anomolous Cu values, therefore unreasonable to determine.

B. Cumulative mortality during toxicity test C.



C. Reasons for rejecting tests A and B.

The LC_{50}'s as determined by tests A and B were somewhat lower than the results obtained by test C (Appendix VIIIA). Various factors may have contributed to this including:

(i) The fish for both tests A and B had not been treated for fin rot (Table 1) which may have contributed to an increased sensitivity to copper due to illness.

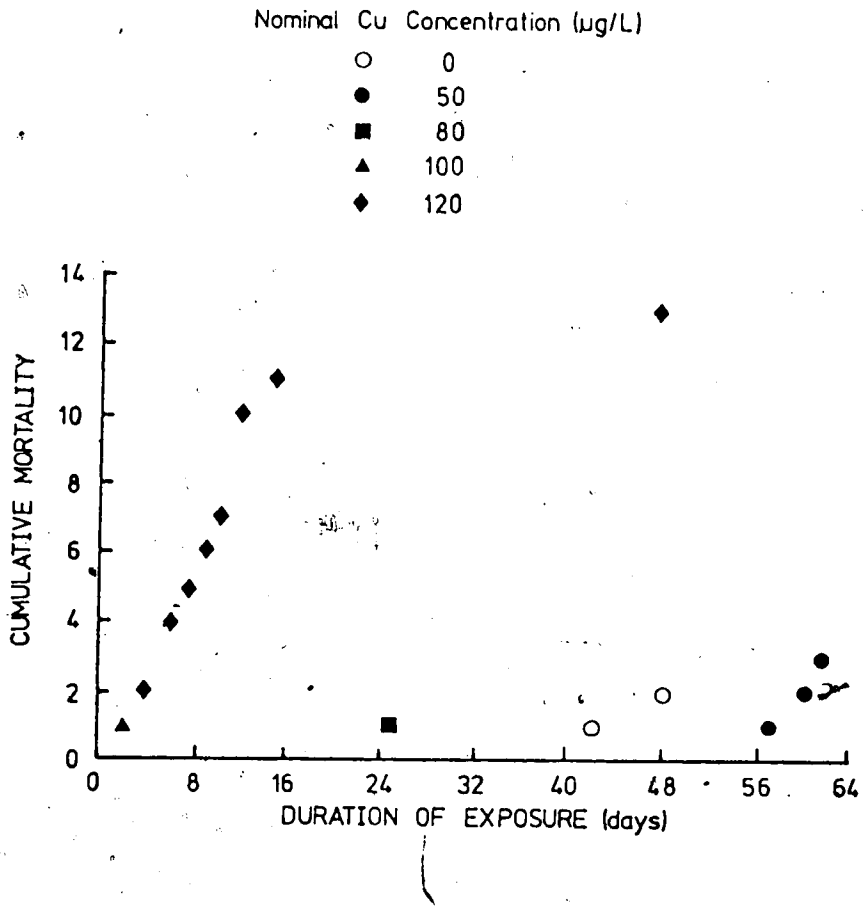
(ii) The fish used in tests A and B were approximately 30% smaller (by weight) than the fish used in test C (Appendix II). Some researchers have noted that smaller trout are more sensitive to copper toxicity (Howarth and Sprague, 1978) while others (Spear and Anderson, 1975) have found that sensitivity of trout to copper was not significantly different over a wide range of body weights. Further experiments would be required to determine if a 30% size difference would be significant for rainbow trout under the testing conditions used in the present study.

(iii) The fish in test A had been added directly to the copper solutions (Table 1); thus, the fish were exposed to a handling stress in conjunction with the copper stress. The occurrance of handling stress in fish has been documented by several researchers (e.g. Wedemeyer, 1972; Mazeaud *et al.*, 1977).

(iv) The fish in test B were probably exposed to reduced oxygen levels for several hours during the test (Materials and Methods). Reduced levels of oxygen have been shown to increase the toxicity of pollutants to fish (Cairns and Scheier, 1957; Lloyd, 1961). However, further experimentation would be needed to determine if a relatively short-term drop in oxygen levels would significantly change the LC_{50} of copper to trout.

Thus, for the above-stated reasons, tests A and B were not considered reliable estimates of the LC_{50} of copper to rainbow trout.

Appendix IX. Cumulative mortality during chronic exposure of rainbow trout to copper, series B.



Appendix X. Chronic exposure of rainbow trout to copper, series A: results and discussion.

The plasma concentrations of Cl^-, Na^+, K^+ and glucose were measured from rainbow trout exposed to concentrations of copper ranging from 0 to 80 µg/L Cu (see Materials and Methods). The data obtained from fish in this series could not be considered with the data from the fish in series B for three reasons:

(i) The trout used for the 20 and the 50 µg/L Cu exposures had not been treated for fin rot (Materials and Methods, Table 2). Over 95% of these fish developed fin rot during exposure to copper, while less than 10% of the treated fish (in both series A and series B) developed the disease during exposure to copper. The fins of the untreated fish were frayed by more than 1 mm in about 40% of the cases while the fins of treated fish which had a re-occurrance of the disease were rarely frayed by more than 1 mm.

(ii) The blood from most of the fish sampled in series A was withdrawn with syringes containing liquid heparin (see Materials and Methods) which diluted the blood sample 1-5%. Since the dilution factor was not constant and could not be determined for each individual sample, some of the changes in the levels of plasma ions or of plasma glucose may be a result of technique rather than copper.

(iii) Significant statistical interactions ($p<0.05$) occurred between the data of the two series. According to Sokal and Rohlf (1969), interactions may occur due to synergism, interference or aberrent replicates. Because of the technique and treatment differences between the two series, the interaction was likely due to aberrent replicates.

The results of the series A chronic exposure experiment are presented in this Appendix (Figures X-1 to X-5 and Tables X-1 to X-3).

The data were statistically analyzed by means of a Hodges-Lehmann Conditional Rank Test (Table X-1) and a Kruskal-Wallis one-way Analysis of Variance (Table X-2). If significant ($p<0.05$) interactions were noted between concentration and day of exposure for a particular parameter the Hodges-Lehmann test was not used. Correlation coefficients between the plasma glucose levels and the plasma ion levels were also calculated (Table X-3).

Since most of the fish used in the 20 and 50 µg/L Cu exposure were diseased, the following discussion will be based only on the results from the fish exposed to 0 and 80 µg/L Cu.

Significant differences between the control and the 80 µg/L Cu-exposed fish occurred only with the measured levels of plasma glucose (Table X-1B). The

levels of glucose in the plasma of the copper-exposed fish were higher than the levels measured in the 0 µg/L Cu-exposed fish (Figure X-4), indicating that exposure to 80 µg/L Cu stressed the fish.

When the blood samples taken with dry syringes were omitted (i.e., 0 µg/L Cu - days 32, 48 and 64; and 80 µg/L Cu - days 48 and 64), no significant differences over time occurred with any of the measured parameters (Table X-2) when the data from each concentration were analyzed separately. When the data from the 0 and the 80 µg/L Cu-exposed fish were considered together, a significant difference ($p<0.05$) over time was observed for plasma glucose (Table X-1B). Since the variations in plasma glucose levels between the days of exposure were similar for both the 0 and the 80 µg/L Cu exposures (Figure X-4), it is suspected that an unspecified stress or technique problem may have caused the variation.

During the 80 µg/L Cu exposure of trout in series B, significant changes ($p<0.05$) were found in the plasma levels of Cl^- and K^+ while changes over time for these two parameters were not found during series A. The changes in K^+ during series B were questioned (see Results) due to the initial difference in plasma K^+ levels of the fish used for the 80 µg/L Cu exposure compared with the levels of the 0 µg/L Cu-exposed fish. However, the fact that significant changes over time

occurred in some parameters measured from the series B fish and not for fish sampled from series A may indicate that 80 µg/L Cu (= 0.8 toxic units; one toxic unit equals the LC_{50} - Sprague, 1970) is near the threshold for Cu to influence plasma ion levels and that the lack of significant changes in series A may be partially the result of error introduced by differential dilution of the blood samples. A significant negative correlation ($p<0.05$) occurred between plasma glucose and plasma chloride when the results from the 0 and the 80 µg/L Cu-exposed fish were combined (Table X-3). A similar correlation ($p=0.001$) was noted for the 0 and 80 µg/L Cu-exposed fish from series B (Spearman's $r = -0.452$) over the same duration of exposure (i.e., days 0-16). Thus, evidence of a relationship between stress and the plasma chloride levels of copper-exposed fish was found in both series of the chronic exposure experiment.

A positive correlation ($p<0.05$) occurred between plasma glucose and plasma sodium at 80 µg/L Cu (Table X-3), while a negative correlation ($p=0.001$) occurred for fish exposed to a higher concentration of copper (120 µg/L Cu, series B - Figure 9). No correlation was found between these two parameters at the 80 µg/L Cu exposure during series B. Since plasma glucose levels were generally higher with Cu exposure (see Figures X-4 and 8 and Christensen *et al.*, 1972) and plasma sodium levels were generally lower with copper exposure

(see Figures X-2, 6 and Courtois, 1976) compared with levels measured from unexposed fish, the positive correlation that occurred with the 80 µg/L Cu-exposed fish in series A was likely a type II error as defined by Sokal and Rohlf (1969).

The positive correlations noted between plasma glucose and plasma potassium was not confirmed by the results in series B. Further research would be required to substantiate such a correlation.

Figure X-1. Plasma chloride values (mean ± S.E.M.) of trout chronically exposed to copper, series A. Except where indicated each point represents the mean value for 5 fish. The open figures (◇,△,○,□) represent blood samples taken with syringes containing liquid heparin. The closed figures (◆,▲,■) represent the blood samples taken with syringes containing air-dried heparin.

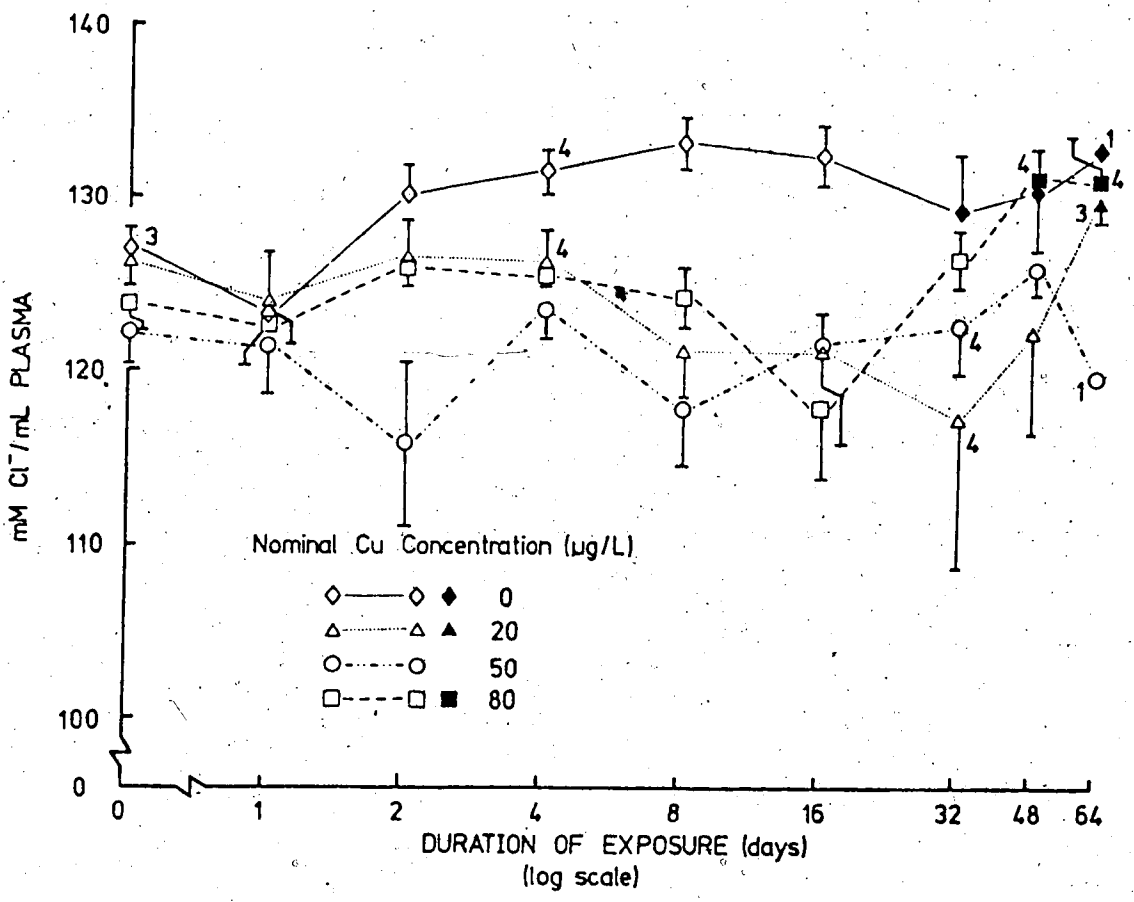


Figure X-2. Plasma sodium values (mean ± S.E.M.) of trout chronically exposed to copper, series A. Except where indicated each point represents the mean value for 5 fish. The open figures (◇,△,○,□) represent blood samples taken with syringes containing liquid heparin. The closed figures (◆,▲,■) represent the blood samples taken with syringes containing air-dried heparin.

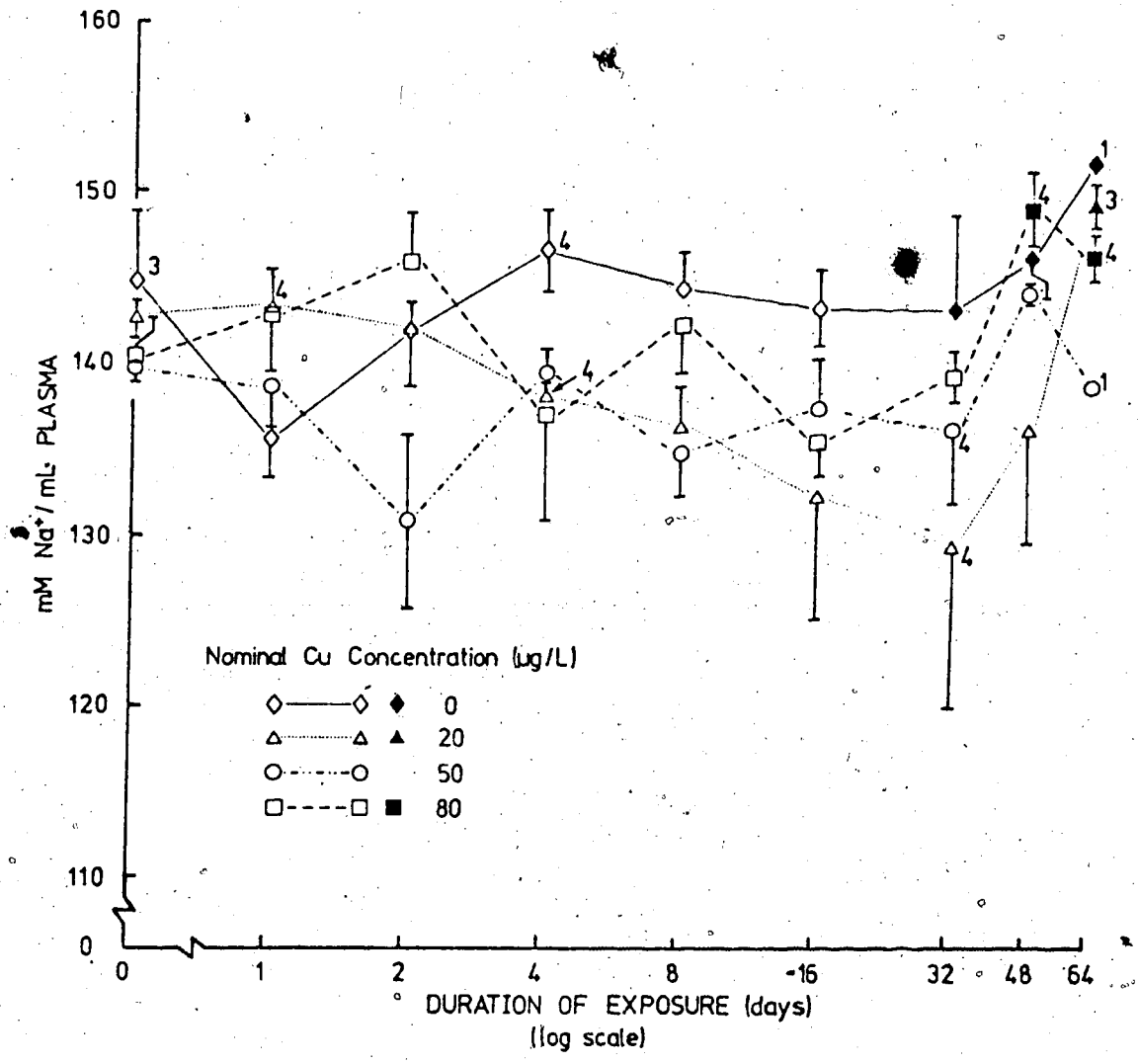


Figure X-3. Plasma potassium values (mean ± S.E.M.) of trout chronically exposed to copper, series A. Except where indicated each point represents the mean value for 5 fish. The open figures (◇,△,○,□) represent blood samples taken with syringes containing liquid heparin. The closed figures (◆,▲,■) represent the blood samples taken with syringes containing air-dried heparin.

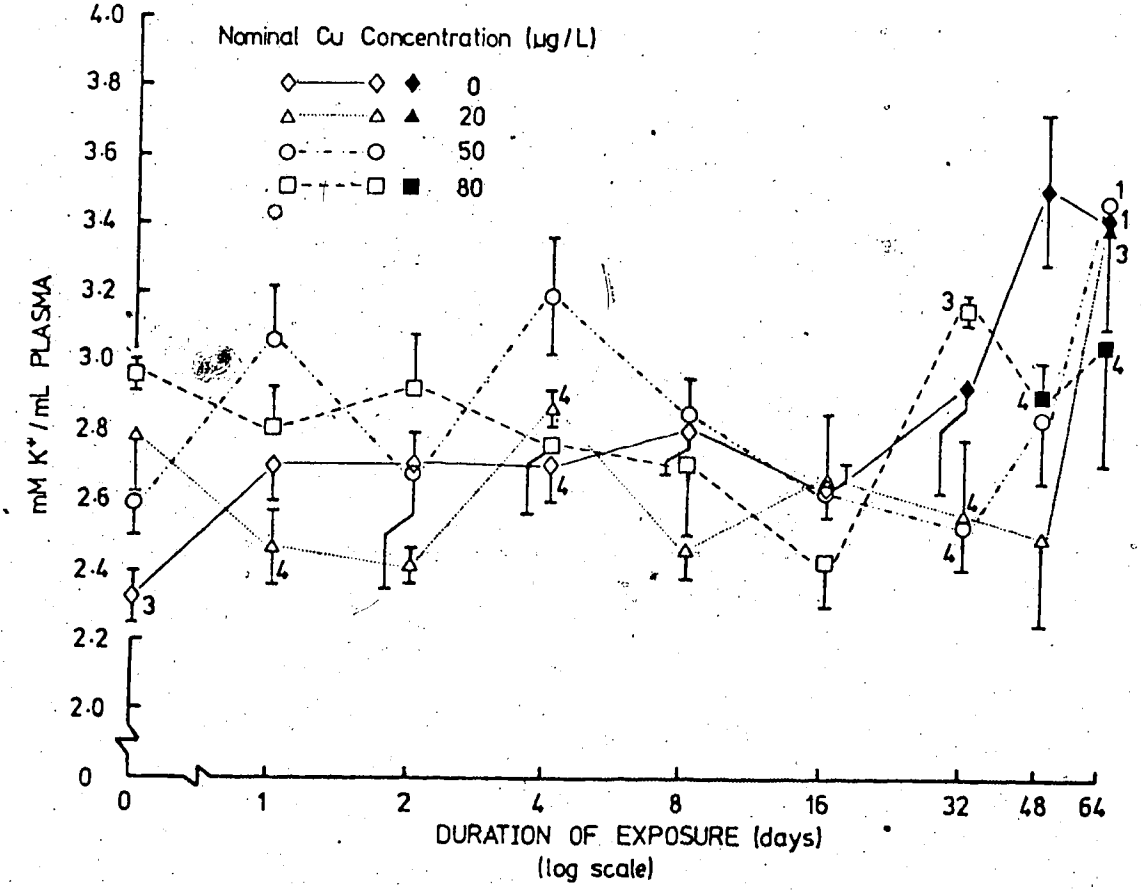


Figure X-4. Plasma glucose values (mean ± S.E.M.) of trout chronically exposed to copper, series A. Except where indicated each point represents the mean value for 5 fish. The open figures (◇,△,○,□) represent blood samples taken with syringes containing liquid heparin. The closed figures (◆,▲,■) represent the blood samples taken with syringes containing air-dried heparin.

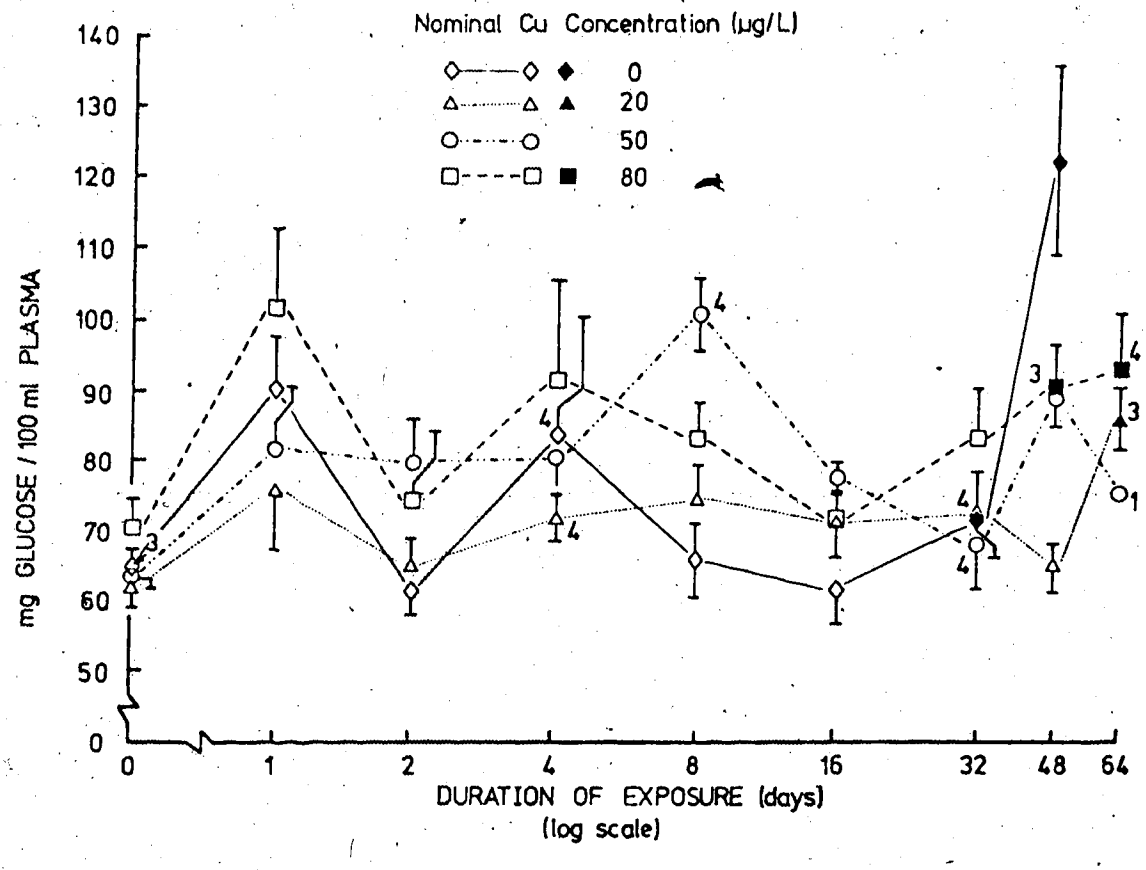


Figure X-5. Cumulative mortality during chronic exposure of rainbow trout to copper. Series A.

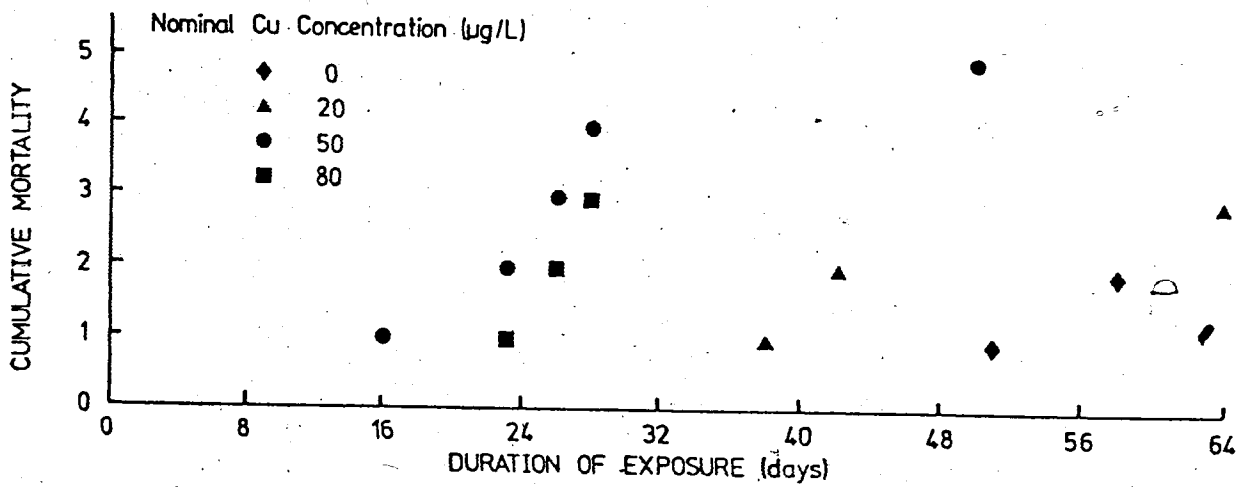


Table X-1. Results of the Hodges-Lehman Conditional Rank Test (Marascuilo and McSweeney, 1977) to determine significant differences in plasma parameters between concentrations of copper and between days of exposure, series A.

A. All series A data

	Plasma Parameter			
	chloride		sodium	
	Cu conc.(µg/L)	day	Cu conc.(µg/L)	day
Hodges-Lehmann Conditional Rank Test	***	NS	**	**
Groups significantly different ($p<0.05$)[1]	0 + 20 0 + 50 0 + 80	NS	0 + 50	NS

B. Data from 0 and 80 µg/L Cu exposures only.

	Plasma Parameter					
	sodium		potassium		glucose	
	Cu conc. (µg/L)	day	Cu conc. (µg/L)	day	Cu conc. (µg/L)	day
Hodges-Lehman Conditional Rank Test	NS NS	NS[2] NS[3]	NS NS	* NS	* **	*** *
Groups significantly different ($p<0.05$)[1]	NS NS	NS NS	NS NS	NS NS	NS 0 + 80	NS NS

[1]Pairwise comparisons based on mean ranks, [2]Results based on data from all days,

[3]Results based on data from days 0-16. The data from blood samples taken with dry syringes were omitted.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table X-2. Results of the Kruskal-Wallis Analysis of Variance (Marascuilo and McSweeney, 1977) to determine significant differences over time in plasma parameters of trout exposed to copper. Series A.

Statistical Test	Plasma Parameters and Copper Concentrations (µg/L)															
	Chloride				Sodium				Potassium				Glucose			
	0	20	50	80	0	20	50	80	0	20	50	80	0	20	50	80
Kruskal-Wallis One-Way Analysis of Variance	NS	NS	NS	NS	NS	* (NS)[2]	NS	NS	* (NS)	* (NS)	*	NS	* (NS)	NS	*	NS
Days Significantly Different ($p < 0.05$)[1]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1] Pairwise comparisons based on mean ranks,

[2] Significance in brackets refers to the significance of the data when the blood samples taken with dry syringes were omitted - indicated only if the significance changed.

* $p < 0.05$.

Table X-3. Correlation coefficients (Spearman's r) between plasma glucose and the plasma ions. Series A.

Plasma Ion	Copper Concentration (µg/L) 0	20	50	80	0 and 80 combined
Chloride	NS	** (0.398)[1]	NS	NS	NS *[2] (-0.250)
Sodium	NS	NS	NS	* (0.358)	NS NS[2]
Potassium	** (0.426)	** (0.479)	** (0.478)	NS	** (0.316) NS[2]

[1]Spearman's r - indicated when significant,

[2]Data from blood samples taken with dry syringes omitted (i.e., days 32, 48 and 64),

*$p < 0.05$, **$p < 0.01$.

Appendix XI. Comparison of the histological ratios measured from regions A, B and C[1] of the gill filaments. The results from the three regions of each gill filament were compared in pairs (i.e., A *vs* B, A *vs* C and B *vs* C). t-tests (Sokal and Rohlf, 1969) were used to determine which pairs were significantly different. The number of pairs of results which were significantly different for each parameter is listed in the table.

Nominal Cu Concentration (µg/L)	Histological[2] ratio	Paired results differing significantly[3] A:B		A:C		B:C	
		p<0.01	p<0.05	p<0.01	p<0.05	p<0.01	p<0.05
0	V_{BC}/V_{PS}	0	1	1	1	1	2
	$V_{EP}/V_{SL'}$	0	0	0	0	1	0
	S_I/S_O	0	0	3	4	2	4
120	V_{BC}/V_{PS}	1	1	0	1	1	1
	$V_{EP}/V_{SL'}$	0	0	1	0	1	1
	S_I/S_O	1	1	3	4	4	4

[1]Region A = proximal third; region B = middle third, region C = distal third.

[2]V_{BC}/V_{PS} = volume of blood channel spaces relative to the pillar system,

$V_{EP}/V_{SL'}$ = volume of the epithelia relative to the secondary lamellae (including the adjacent epithelia of the primary lamella),

S_I/S_O = surface area of the epithelial basement membrane relative to the outer surface area of the epithelia. See Figure 2.

[3]N = 7 for each group.

Appendix XII. The effect of tricaine methane sulphonate on gill histology.

An attempt was made to determine the cause of the separation of the gill epithelia from the pillar system of the secondary lamellae. The pH of the anesthetic solution used to kill the fish before removal of the gills was quite low (approx. 3.6) and extreme pH's have been reported to cause epithelial separation in fish gills (Daye and Garside, 1976). Thus, an experiment was carried out to determine if the anesthetic solution used during the course of the chronic exposure to copper experiment could have caused the lifting of the epithelium found in most of the gill sections.

I. Material and Methods

Four fish (35.83 ± 2.51 g) were placed in a 0.05% solution of tricaine methane sulphonate (Kent Laboratories), using synthetic freshwater (see Materials and Methods) as a diluent, for 10 minutes. The solution was lethal to the fish within the time of exposure and had a pH of 3.56. The gills of these fish and of four control fish (35.21 ± 3.36 g), held only in synthetic freshwater (pH 7.39), were excised and fixed as described below. The control fish were killed by severing the spinal cord behind the head.

The gills of two experimental fish and of two control fish were fixed in 2.5% gluteraldehyde and 0.2 M phosphate buffer (Cloney and Florey, 1968). Some of the fixative was poured over the gills before they were excised. The second gill arch was removed for further fixation. The gill samples were then post-fixed in 4% OsO_4 (Ludd Chemicals Co.), dehydrated in a graded series of alcohol and embedded in Epon plastic (Shell Chemical Co.). Single filaments were sectioned to a thickness of 1 µm, mounted, and stained with Richardson's methylene blue stain (Richardson *et al.*, 1960).

The gills of the remaining four fish (two experimentals and two controls) were fixed in F.A.A., embedded in Paraplast, sectioned, mounted and stained with Herovici's stain in the same manner as used in the chronic exposure to copper experiment (see Materials and Methods, and Appendix VII).

II. Results

As shown by Figures XII-1 and XII-2 no noticeable differences were found between the gills of the control fish and the gills of the fish killed in tricaine methane sulphonate. However, lifting of the epithelia did occur in the gills of fish fixed in F.A.A. and embedded in Paraplast (Figure XII-2) while lifting did not occur in the gluteraldehyde-fixed and Epon-embedded gills

Figure XII-1. Effect of tricaine methane sulphonate on the secondary lamellae of rainbow trout gills. Epon-embedded gills. Note lack of epithelial separation from the pillar system in both A and B. EP - epithelium; PS - pillar system; NT - non-tissue space.

A. After exposure to tricaine methane sulphonate

B. Control

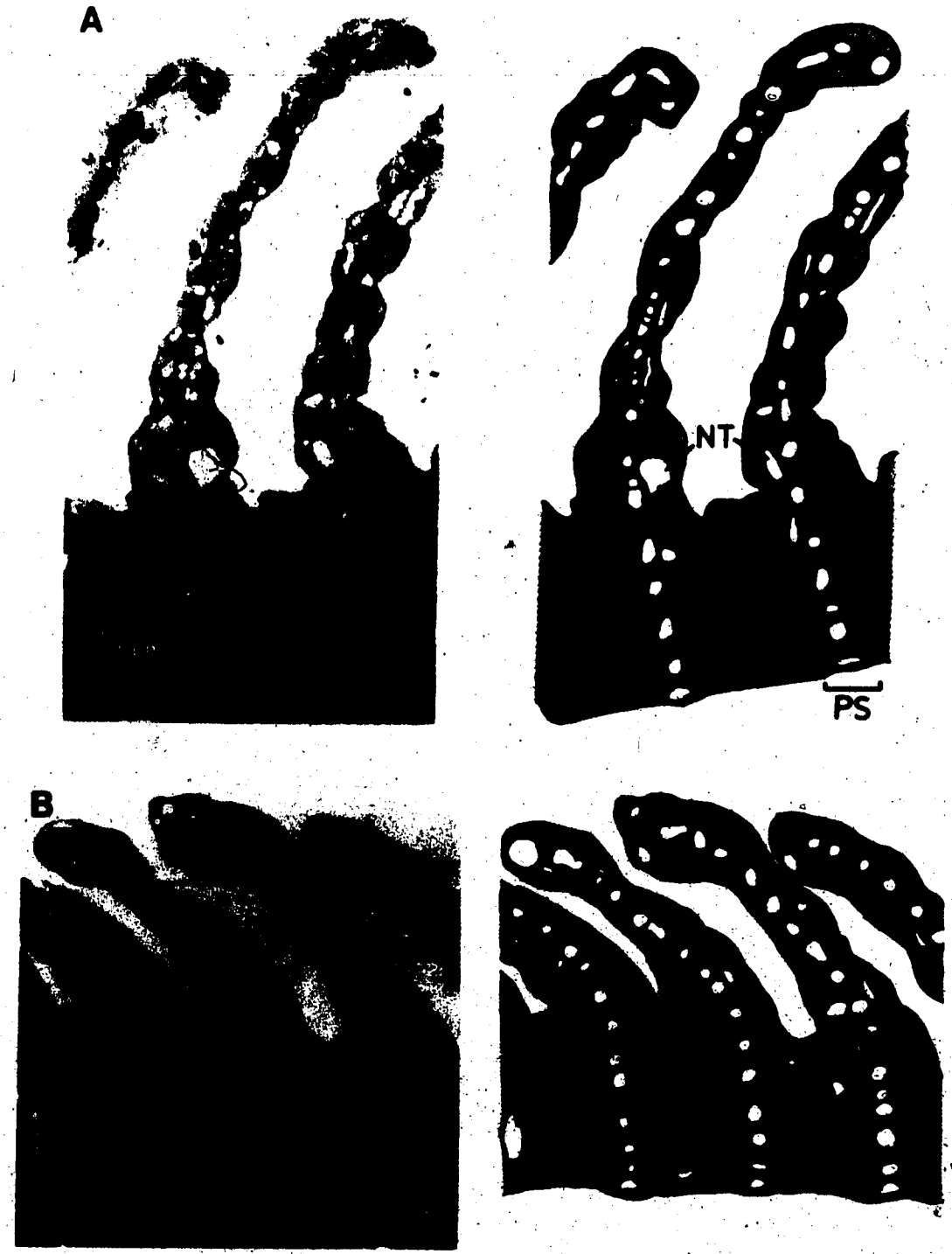
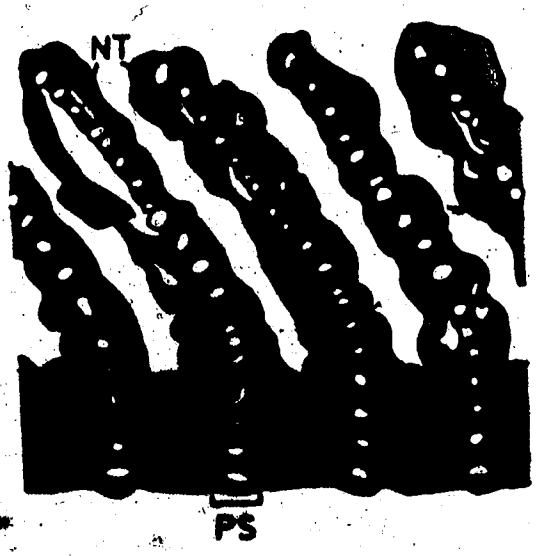

A
NT
PS
B

Figure XII-2. Effect of tricaine methane sulphonate on the secondary lamellae of rainbow trout gills. Paraplast-embedded gills. Note the separation of the epithelium from the pillar system in both A and B. EP - epithelium; PS - pillar system; NT - non-tissue space.

A. Control

B. After exposure to tricaine methane sulphonate


A


NT
PS

(Figure XII-1). Thus, it appears that the anesthetic used to kill the fish was not the cause of the separation of the epithelia from the pillar systems. Further experimentation would be needed to determine which step in the preparation of the slides using F.A.A. fixation would have caused separation of the epithelia.