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Effects of Chemical Variations in Aquatic Environments Vol. III Lead Toxicity to Rainbow Trout and Testing Application Factor Concept

Office of Research and Monitoring
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February 1973

EFFECTS OF CHEMICAL VARIATIONS IN AQUATIC ENVIRONMENTS:

Volume III

Lead Toxicity to Rainbow Trout

and

Testing Application Factor Concept

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ABSTRACT

Four chronic bioassays were conducted to determine the toxicity of lead to rainbow trout. Results obtained from acute and chronic bioassays in hard water (alkalinity 243.1 mg/liter) and soft water (alkalinity 26.4 mg/liter) were used to test the application factor approach as related to different water qualities. The toxicity of lead to rainbow trout in hard water was determined on a total and dissolved lead basis. The 96-hr TL_{50} and "MATC" on a total lead basis were 471 mg/liter and 0.12 to 0.36 mg/liter respectively, which yielded an application factor of .0002 to .0008. Analysis of the free or dissolved lead gave a 96-hr TL_{50} of 1.38 mg/liter and a "MATC" of 0.018 to 0.032 mg/liter, resulting in an application factor of .0130 to .0232. Total and free lead were considered to be the same in soft water. The 18-day TL_{50} and "MATC" obtained from the soft water bioassays were 140 μg/liter and 6.0 to 11.9 μg/liter lead respectively. Computations using the TL_{50} and "MATC" values gave a soft water application factor of .0429 to .0850. The maximum acceptable toxicant concentration ("MATC") was determined in both hard and soft water bioassays on the occurrence of abnormal black tails caused by chronic lead exposure. The application factor approach as related to different water qualities was found to be very promising when lead analysis was limited to the free or dissolved metal and failed when total hard water lead concentrations were used.

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Section I

CONCLUSIONS

Hard Water Bioassay Findings

1. In hard water the mean hardness, M.O. alkalinity and pH was 353.5 mg/liter, 243.1 mg/liter and 8.02 respectively.

2. Lead analysis in hard water is reported as total and free lead.

3. A static acute bioassay gave a 96-hr (50% tolerance limit) TL_{50} of 471 mg/liter total lead. The maximum acceptable toxicant concentration ("MATC") from the hard water chronic bioassay was between 0.12 and 0.36 mg/liter total lead. The acute and chronic ("MATC") total lead concentrations give an application factor between .0002 and .0008. The "MATC" is based on the occurrence of black tails which were caused by lead. Lead-attributed mortalities in the chronic bioassay occurred in the high concentration (3.24 mg/liter total lead).

4. On a free lead basis, the static hard water acute bioassay gave a 96-hr (50% tolerance limit) TL_{50} of 1.38 mg/liter. The "MATC" of the chronic bioassay based on black tails, was between 0.018 and 0.032 mg/liter free lead. The hard water free lead application factor lies between .0130 and .0232. Lead-attributed mortalities occurred at a free lead concentration of 0.064 mg/liter in the high concentration aquarium.

5. Physical abnormalities consisted of black tails and spinal curvatures (lordoscoliosis).

Soft Water Bioassay Findings

6. In soft water the mean hardness, M. O. alkalinity and pH were 27.2 mg/liter, 26.4 mg/liter and 6.88 respectively.

7. Lead analysis in soft water is considered as free lead. A flow-through acute bioassay gave an 18-day (50% tolerance limit) TL_{50} of 140 µg/liter lead. A "MATC" based on black tails, was found to be between 11.9 and 6.0 µg/liter lead. The soft water application factor for lead lies between .0429 and .0859. Lead-attributed mortalities with fry and fingerling fish occurred in the high concentration aquarium (95.2 µg/liter).

8. Three-year-old brood fish of the soft water reproduction bioassay yielded viable eggs and fry from all but the lowest toxicant concentration. The high lead concentration of this experiment was 27.9 µg/liter.

9. Two-year-old rainbow trout proved to be inadequate for use in determining possible effects of lead on reproduction. Only 24% of the 63 females examined were found to be reproductively mature.

10. No growth differences attributable to lead were found in either the hard or soft water growth bioassays.

11. Hematocrit and hemoglobin determinations did not provide a consistent statistical difference in either the hard or soft water growth bioassays. However, the tap water F_1 generation reproduction bioassay in which the lead concentration was increased, gave a statistically lower hematocrit value for the high concentration (95.2 μg/liter Pb).

Testing of the Application Factor Approach as Applied to Water Qualities

12. Using free or dissolved lead analyses, the application factor approach succeeded in estimating "MATC" levels in both hard and soft water experiments. Therefore, it appears that the application factor approach as applied to different water qualities does have promise when the metal analysis is limited to that of measuring the dissolved metal.

13. On a total (nominal) lead basis, multiplying the soft water application factor by hard water, total lead TL_{50} concentration failed to approximate the hard water "MATC". The converse relationship of multiplying the hard water application factor by the soft water TL_{50} also failed to approximate the soft water "MATC". Therefore, on the basis of total lead, the use of an application factor for estimating the "MATC" of a water for which only acute tests can be made, failed.

14. The method of analysis, free versus total metal analysis, appears to pose a problem only where a particular metal becomes complexed or suspended in a specific water quality or type.

Section II

RECOMMENDATIONS

More information needs to be obtained to learn those factors that may affect growth in chronic bioassays if toxicant effects are to be properly assessed. Our studies indicate that four factors should receive particular concern. (1) Position effects: Aquaria should be positioned in such a manner as to provide equivalent light to all tanks because of plankton growths which could act as a supplemental food source. The possibility of temperature difference between aquaria in a particular experiment must be minimized. If aquaria are placed on stands with one higher than another, stratified temperature zones in the room cause different aquarium temperatures. Possible temperature differences caused by heat registers, windows, doorways and electrical motors in a room must be considered. Aquaria for a particular experiment need to be positioned to minimize and provide equal exposure to human activities. (2) Equal available space: The available space per gram of fish in a set of experimental aquaria should be the same for any particular bioassay. This may be accomplished through the use of dividers or by adjusting water volumes by aquarium stand pipe height. (3) Feeding rates adjusted daily: Feeding rates based on total weight of fish per aquarium should be adjusted daily. The total weight can be determined bimonthly and adjusted daily from a computerized growth rate projection. (4) Individual marked fish: Toxicant related growth effects can be assessed most reliably by following the growth of individually marked fish. Individualized marking by cold branding was preferred in this study.

Chronic bioassay beginning with eggs obtained from one female for each concentration should be avoided. Size and genetic differences of such eggs can easily mask any toxicant-related effects. The problem is compounded in that eggs obtained from individual females will most likely hatch on different dates, thus greatly complicating data collection and handling. These complications can be avoided by using eggs collected from a large number of females that were spawned on the same date. Experimental results also show that use of 2-year-old rainbow trout as brood fish is impractical for studying toxicant-related effects on reproduction. Only 24% of the rainbow trout females encountered in this study were reproductively mature as 2-year-old fish.

Results from this study support the application factor concept as related to different water qualities only where analysis of the soluble or free metal was employed. Where heavy metals form complexes in natural waters, some standardization of water sample collection and method of analysis will be required if the application factor approach is to find widespread use. A standardization of metal analysis for free metal ions will also be required for acute and chronic bioassay experiments where the complexing of a particular heavy metal occurs.

Considerable research is needed on heavy metals, such as lead, which become complexed and/or precipitated in water of different hardness and/or alkalinity. The problems associated with such metals and attempts to estimate "MATC" levels are numerous and mostly unresolved.

Section III

INTRODUCTION

The effect of heavy metals and their accumulation in the various tropic levels of our environment is a source of much concern today. Lead is one metal that is obtaining increased prominence and a source of much public concern (Hall, 1972). The atmosphere,coastal and inland waters adjacent to highly populated and industrialized areas show an alarmingly high rate in increased lead concentrations over the past 30 years (Chow and Earl, 1970; Hall, 1972). Lead is a significant pollutant in many of our nation's water-ways because of its indiscriminate release in many industrial effluents.

Lead warrants particular interest in Colorado because of the activities of several mining and milling operations which release it to the aquatic environment in effluent waters. It is becoming quite apparent that the relative solubility of some metals in "natural" waters has little relevance regarding their possible toxic effects on the aquatic and terrestrial environments. Lead is a metal previously thought to be of little concern because of its relative insolubility in all but the softest of "natural" waters. Toxic modes other than transference or absorption across the gill membrane are greatly in need of investigation.

An abundance of toxicological data on a multitude of toxicants has been collected and reported during the past century (McKee and Wolf, 1963). Most of this data have been reported as the median tolerance limit (TL_m or TL_{50}). Many, by multiplying the TL_{50} value by some arbitrary fraction, have erroneously interpreted these findings as being those concentrations at which fish life can survive and reproduce. Frequently bioassay data is of little value due to the lack of accompanying water quality information.

Little information has been available on the effect of long-term, sublethal exposures of fish to toxic agents. The effect of toxicants on reproduction is almost unknown except for a few elements. Information on the sensitivity of different life stages of fish to a particular toxicant is generally lacking. Consequently, estimates of safe concentrations based on acute toxicity studies are very difficult to make and frequently erroneous. There exists a vital need for long-term or chronic toxicity studies. During the past few years, some headway has been made in obtaining such information (Arthur, 1970 - LAS detergent; Buhler, Rasmusson and Shanks, 1969 - DDT; Brungs, 1969 - Zinc; McKim and Benoit, 1971 - Copper; Mount, 1962 - Endrin; Mount, 1968 - Copper; Mount and Stephan, 1967a - Cadmium; Mount and Stephan, 1969 - Copper; and Pickering and Thatcher, 1970 - LAS detergent). However, the problem regarding methods by which laboratory toxicity finds might be applied to a multitude of aquatic species and water types must be resolved.

Henderson (1957) discussed a number of constituents involved in developing "application factors" whereby laboratory toxicity studies could be used in determining permissible concentration of toxic substances in the aquatic environment. Mount and Stephan (1967b) proposed the development of an application factor based on dividing the "maximum acceptable toxicant concentration" -- MATC (i.e., that concentration obtained from a chronic bioassay at which no inhibiting effect on growth or reproduction occurred) by the 48-hr or 96-hr TL_{50} concentration for a particular toxicant. The fraction or application factor so obtained is then multiplied by a TL_{50} value found for fish and waters on which long-term testing cannot be performed. The resulting concentration hypothetically establishes the maximum permissible concentration of a particular toxicant in other water quality types containing different fish species. It has been proposed that such an application factor will hold true irrespective of fish species or water quality involved. The authenticity of such factors needs to be established.

Brungs, 1969, further defined the "laboratory fish production index" (LFPI) proposed by Mount and Stephan (1967b) as reflecting toxicant effects on reproduction, growth, spawning behavior, egg viability and fry survival. "The highest observed toxicant concentration that has no effect on these biological factors based on a continuous chronic exposure is termed the maximum acceptable concentration of that toxicant", (MATC). The reproductively mature fish in the present study were artificially spawned and therefore failed to adhere strictly to the criteria outlined above. The further use of the MATC in this report will be in quotation marks (i.e. "MATC") to signify the absence of spawning behavior data.

This study had two primary objectives. (1) to determine acute and chronic toxicities of lead to rainbow trout and (2) to determine the validity of the application factor concept using two distinctly different water qualities. The two water quality types used are: (1) a hard well water (353 mg/liter total hardness) from a 20 - ft well located at the Colorado Game, Fish and Parks Research Center and (2) a soft dechlorinated tap water (carbon filtered) with a total hardness of 27 mg/liter.

Chronic toxicity of lead was conducted using two life stages of rainbow trout. Three-inch rainbow fingerlings were used to measure growth effects and mature, 2-year-old rainbow trout (spawned as 3-year-olds) were used to measure the effect of chronic lead toxicity on reproduction, egg viability and fry growth.

Application factors were calculated for both soft and hard waters. This entailed dividing the "MATC" of the chronic bioassays by the 96-hr TL_{50} for each of the respective waters. The factors or fractions so calculated were used to determine the validity of the application factor concept. This was accomplished by multiplying the application factor obtained from the acute and chronic bioassays for the hard water by the 96-hr TL_{50} value obtained from the acute bioassay performed in dechlorinated soft water. The resulting "MATC' should equate or approximate the "MATC" value obtained from the chronic bioassay in soft water. The converse relationship for hard water should also occur if the application factor concept is upheld.

Previous Lead Experimentation Applicable to the Present Study

Carpenter (1927 and 1930) found that one part lead in three million parts of water (0.3 mg/liter) was lethal to minnows, sticklebacks and trout in distilled water. The speed of the toxic reaction is dependent on the total quantity of metallic ion present, as well as upon the actual concentration. Fish sensitivity to lead varies inversely to fish size and weight, and varies directly, consistent with Van 't Hoff's Rule, to water temperature. She further states that the toxic action to be purely external in process, chemical in type, and mechanical in effect; i.e., a colloidal substance forms on the gill epithelium and eventually causes death by suffocation.

Dawson (1935) reported that the surface mucous, described by Carpenter, did not constitute an effective barrier to the absorption of lead. In his experiments, brown bullheads, Ameiurus nebulosus, survived 16-183 days in tap water renewed at 2-day intervals with 27.3 mg/liter of lead, but observations on the peripheral blood demonstrated direct injury to the erythrocytes followed by a mild regenerative response with the eventual development of pronounced secondary anemia.

Jones (1938) with soft water static bioassays reported lethal concentration limits of 1 mg/liter lead (as lead nitrate) with the minnow, Gasterosteus sp. Lower lethal concentrations were obtained by periodically renewing the lead solution. He also found that younger fish are more sensitive to the toxic action of lead. The addition of calcium salts (as nitrate or chloride) was found to reduce the toxicity of lead. Lloyd (1965) reported a reduced toxicity of lead with increase in water hardness. Rainbow trout were found to be more susceptible to the toxicity of lead when the dissolved oxygen concentration of the water was reduced (Lloyd, 1961 and 1965).

Lloyd (1965) has postulated that the acute toxicity of lead to fish results from attaining a lethal threshold concentration of a heavy metal at the gill surface. If the rate at which the heavy metal ions enter the gill epithelium is greater than the rate at which they are removed into the blood stream, then a buildup will occur, and the fish will die. The fish will survive if the converse relationship exists. Haider (1964) reported that acute toxicity causes death through erosion of the gill epithelium and a resulting suffocation. He further states that chronic lead poisoning amounts to a resorption of the toxicant, apparently through the gills, slowly resulting in functional damage of the inner organs.

Behavioral symptoms of fish chemically exposed to lead were described by Haider (1964) These are: Fish were exposed to lead acetate in low concentrations which were increased over time. The fish showed a gradual darkening over their whole body. This occurred very quickly (about 2 hr) when fish were injected with lead. A sharply defined darkening occurred

to the caudal part of the body when lead was injected into the top of the ventral fins. Fish that were daily fed lead in their diet also became completely dark colored. Irregularity in breathing was occasionally observed but over time this occurred more frequently. It became difficult for the fish to maintain their equilibrium and were seen to tip over into a slanting position and move jerkily through the aquarium. The fish were frequently observed lying on the aquarium bottom with their fins spread out. From time to time wave-type muscular movements occurred over the body. The fish declined food. The slime secretions reported by Carpenter (1927 and 1930) were not observed.

Dorfman and Whitworth (1969) reported on the effects of fluctuations of lead, temperature and dissolved oxygen on the growth of brook trout, Salvelinus fontinalis. All treatment combinations that included a lead concentration of 25 mg/liter, administered once a day as a slug dose, reduced growth. Treatment combinations that included 15 mg/liter and 10 mg/liter of lead administered as a slug dose once a day showed no apparent effect on growth.

Information on the toxicity of various lead salts have been summarized by McKee and Wolf (1963). Concentrations of 0.1 to 240 mg/liter of lead for different water qualities have been reported as acutely toxic to fish as a 96-hr TL_{50} concentration. Pickering and Henderson (1965) conducted static bioassays in soft (alkalinity - 18 mg/liter, hardness - 20 mg/liter) and hard (alkalinity - 300 mg/liter, hardness - 360 mg/liter) waters. Using lead chloride in soft and hard waters, the 96-hr TL_{50} for fathead minnows was 5.58 mg/liter of lead and 482 mg/liter of lead respectively. Similar tests in soft and hard water on bluegills give a 96-hr TL_{50} of 25.9 and 442 mg/liter of lead respectively.

Henderson (unpublished data) compared TL_{50} toxicity concentrations for static versus constant-flow bioassays. Using lead chloride in soft water (pH - 7.4, alkalinity - 18 mg/liter and hardness - 20 mg/liter) with fathead minnows as the test fish, he determined the 96-hr static TL_{50} to be 5.6 mg/liter of lead as compared to a 96-hr constant-flow TL_{50} of 0.97 mg/liter of lead. Using a constant-flow bioassay, Henderson found TL_{50} concentrations at the indicated time intervals to be, in mg/liter of lead: 1.1 (48 hr), 0.97 (96 hr), 0.75 (5 days), 0.40 (10 days), 0.31 (20 days) and 0.31 (35 days).

Section IV

METHODS

EXPERIMENTAL CONDITIONS

Two different water qualities were used for the bioassay experiments. Hard water with a mean hardness of 353.5 mg/liter and a mean M.O. alkalinity of 243.1 mg/liter was obtained from a 20-ft well located at the Research Center for the Colorado Division of Game, Fish and Parks. Fort Collins city water with a mean hardness of 27.2 mg/liter and a mean alkalinity of 26.4 mg/liter was dechlorinated by passage through a Hayward Model S-40 Sand Filter (modified) containing 12 cubic ft of activated carbon. The filter was a Maycor epoxy-lined carbon steel vessel with PVC (polyvinylchloride) and stainless steel internal components. Piping from the filter and within the chronic and acute bioassay rooms was PVC.

The chronic bioassays were conducted at the ambient temperature of Fort Collins city water which is obtained from the Cache La Poudre River. The well water maintains a year-round temperature of 15°C and was cooled to the ambient tap water temperature using a "Min-o-cool" fiberglass tank with two 1-horsepower refrigeration units. Soft water was cooled to the ambient hard water temperature when summer water temperatures exceeded 15°C. Temperature adjustments were performed on a weekly basis when required. Temperatures were monitored in the soft and hard water control aquaria by Foxboro recording thermometers.

Chronic bioassays were performed in 325-liter, glass aquaria (142 cm x 47 cm x 50 cm) with plexiglass covers. Each aquarium was aerated with two 6-inch airstones. An initial aquaria hygiene problem was alleviated through the use of a settling trap, similar to an Imhoff cone, which was mounted outside each aquarium, for removal of settleable solids. Supernatant water from the settling trap was siphoned to another container packed with a dacron polyester fiber through which the water was pumped for removal of suspended matter and returned to the aquarium (Citation in preparation). Aquaria walls were brushed two to three times a week to remove algal growths of Achanthes sp. The toxicant delivery system for each bioassay utilized a proportional diluter (Mount and Brungs, 1967) that delivered 2 liters of test water per cycle or approximately 1 liter per 2 min to each exposure aquarium. This rate of delivery gives a 50% water replacement time of about 6.3 hr (Sprague, 1969). Lead nitrate ($PbNO_3$) comprised the stock solution that supplied the diluters. Acute bioassays employed a similar toxicant delivery system and used 35-liter aquaria with each test concentration duplicated.

CHEMICAL METHODS

Water quality analyses were performed weekly on the chronic bioassays. Parameters that were measured include: temperature, conductivity, pH, dissolved oxygen, alkalinity and hardness. Chemical methods were made in accordance with standard methods of the American Public Health Association et al. (1965). Temperature determinations were made with a TRI-R Electric Thermometer. A Beckman Model RB-2 Solu Bridge was used for conductivity measurements. A Corning Model 12 pH Meter gave readings to a hundredth of a pH unit. Dissolved oxygen, alkalinity and hardness were determined by the azide modification method, pH titration to a 4.5 (M.O.) end point, and the EDTA titrimetric method, respectively.

ANALYTICAL METHODS FOR LEAD

First and foremost, it is necessary that one understands that the occurrence of lead in what might be called "natural" water constitutes an extremely complex relationship of a multiplicity of various physical, biological and chemical interactions. The resulting system is not one that can be empirically described at this point in time, and is one which will require an extensive amount of research before a compatible understanding of the system is achieved. (See Appendix A for a more detailed discussion on the complexities associated with analyzing the character of lead in water).

Atomic Absorption

Atomic absorption analysis was performed on water samples collected from all aquaria of the hard water growth bioassay. Analysis of soft water growth and reproduction bioassay samples was limited to the high concentration aquaria because of the detection limit of the equipment. Analyses were also performed on water taken from the two high concentrations of the F_1 generation reproduction bioassay. Weekly grab samples were taken in new 140-ml Nalgene bottles, acidified with three to five drops of "Suprapur" nitric acid (EM Laboratories, Inc.). Analysis was conducted using a Perkin-Elmer 303 Atomic Absorption Spectrophotometer set at a wave length of 2170.0 A or 2833.1 A. A Perkin-Elmer 10 mv Recorder was also used with a noise suppression level setting of three and 10X to 30X scale expansion. Unknown lead concentration levels were determined from a calibration curve constructed from acidified standards prepared in distilled water. (See Appendix B for a further discussion of problems associated with the analysis of lead by atomic absorption spectrophometry).

Pulse Polarography

Water samples from hard water growth, soft water growth and reproduction bioassays were analyzed by pulse polarography from 14 August 1970 to 31 December 1970. Lead concentrations were monitored weekly from composite samples. Twenty-five milliliter water samples were collected daily (Monday through Friday) and pooled in a 140-ml Nalgene bottle which was sealed and returned to the bottom of the aquarium to prevent changes due to CO_2 absorption. Following Friday's sampling, the bottle was stored in a "Whirl Pack" filled with water from the experimental aquarium where it remained until analysis. The time delay for this analysis was a few hours to two months with an average delay of about two weeks. Such delays did not create significant systematic error to affect analytical results. However, changes (were they to occur) would result primarily from pH changes due to loss or increase of carbon dioxide, a consequence that was minimized by the method of sample storage.

The pulse polarographic analyses were conducted using a Melabs Pulse Polarographic Analyzer with an accompanying electrode stand. Because of the availability of instrumentation, some analyses were done using a PAR Model 170 Electrochemical System with a PAR Model 174 Drop Knocker Assembly. The two systems showed no significant difference in analytical results. The indicator electrode, used with both systems, was a dropping mercury electrode from which the drops were mechanically dislodged at 3- to 5-sec intervals. A large mercury pool served as both reference and counter electrode. Reagent grade chemicals were used without further purification.

Analysis procedure for lead concentrations above 10 μg/liter

Under the experimental conditions employed,using 100 mv pulse modulation in the differential mode, the detection limit was approximately 10 μg/liter lead. Solutions with nominal lead concentration above this value were analyzed by first adding 25 ml of the sample to the cell, deaerating for 8 min with prepurified nitrogen, and running the polarogram. Polarograms were similarly run with the addition of 0.1 ml saturated LiCl solution (Ca 10 M), in an attempt to attain a lead signal not obtained directly. Under these conditions the peak for lead reduction to the amalgam occurs at approximately -420 mv. Of the samples analyzed by these two procedures, approximately 60% gave no signal for lead.

Following direct analysis procedures which were largely unsuccessful in obtaining a lead signal, the samples were acidified with a few drops of concentrated HCl (to about pH 2) and a polarogram run immediately. This procedure gave a lead signal for those samples where no signal was obtained

by direct analysis. A small known amount of a lead standard solution was then added to the acidified solutions in an amount sufficient to increase the peak height by about 30%, and a second polarogram was run. Sample concentrations were calculated in the normal manner from the two polarograms using the added lead as an internal standard. Ultimately, all samples with a nominal concentration above 10 μg/liter were acidified immediately prior to analysis. (See Appendix C for a more detailed discussion of the pH problems associated with the analysis of lead by pulse polarography).

Analysis procedure for lead concentrations below 10 μg/liter

Solutions with nominal lead concentrations of less than 10 μg/liter were extracted prior to analysis. The following was added to 50 ml of sample: 5 ml 1% sodium diethyldithiocarbamate, 2.5 ml 1 M sodium citrate,and sufficient HCl to adjust the pH to 11.0. The resulting solution was extracted with 10 ml MIBK (methyl isobutyl ketone). Five milliliters of 0.5 M LiCl in methanol was added to the MIBK extract and the solution diluted to 25 ml with methanol. The methanol solution was then added to the cell, deaerated for 10 min, and a differential pulse polarogram run. Peak heights were compared with those obtained for standard lead solutions subjected to the same procedure. Under these conditions the lead peak occurs at about -370 mv. The detection limit is approximately 2 μg/liter. Some analyses were made by a similar procedure employing an extraction of the 8-hydroxyquinoline complex in NH_3/NH_4Cl buffer. Results for the two extraction procedures were comparable.

Metal Analysis of Feed

Analyses for zinc, copper and lead were made on the feeds used in the lead bioassay experiments. Samples were dried for 24 hrs at 95°C. Sample sizes of 0.5 g and 1.0 g were digested in 1 ml concentrated perchloric and 2 ml concentrated nitric acids using a wet, pressure digestion method (Adrian, 1971). Amount of lead in feed was found to be less than 2.5 μg/g. (See Appendix D for more detailed results).

CHRONIC BIOASSAY METHODS

Hard and Soft Water Growth Bioassays

Fish and toxicant system

Two growth bioassays were initiated on 14 May 1970, one in filtered soft water and the other in hard water. Seventy rainbow trout, averaging 83.3

mm in length and 6.1 g in weight, were placed in each aquaria. These fish were hatched and raised to fingerling size at Bellvue Research Hatchery from eggs obtained from the McCleary rainbow trout strain at Soap Lake, Washington. The fish were graded for size uniformity just prior to being placed in the test aquaria. Fish were acclimated to water quality and the aquarium environment for 2 weeks prior to the introduction of toxicant. The proportional diluter for the soft water bioassay was set at 33.3% dilution ratio with a high nominal concentration of 30 μg/liter lead. Nominal lead concentrations in μg/liter for the soft water growth bioassay were: S-1 (30.0), S-2 (20.0), S-3 (13.3), S-4 (8.9), S-5 (5.9) and S-C (0.0). For the hard water growth bioassay the proportional diluter was set at a 66.6% dilution ratio with resulting nominal lead concentrations in mg/liter of: H-1 (3.24), H-2 (1.08), H-3 (0.36), H-4 (0.12), H-5 (0.04) and H-C (0.00).

Feed and feeding rates

The growth bioassay fish were fed the standard Colorado diet at a feeding rate and pellet size obtained from the modified Cortland feeding chart (Deuel, Haskell and Tunison, 1937). The feeding rate ranged from 7% of body weight per day for 6-g fish to 1.0% for 325-g fish. After 6 months of feeding the Colorado diet, fish condition indicated the possibility of nutritional deficiencies. Consequently, a supplemental feeding of beef liver was initiated weekly (personal communication Dr. Donald Horak, Nutritionist, Colorado Game, Fish and Parks). Feeding rates were adjusted monthly for the first 8 months from the total weight of fish per aquarium obtained using a triple beam balance. Following this period, feeding rates were adjusted every 3 months.

Growth measurements

Ten fish of the initial 70 placed in each aquarium were fin clipped for individual identification. Growth measurements consisting of total lengths (mm) and wet weight (g) were determined on these individuals for the first 3 months of the experiment. It was then decided to discontinue the collecting of growth data from fin-clipped individuals because of the questionable influence of handling on these selected individuals as compared to the remaining unmarked fish in an aquarium. Subsequent to this decision, a random sample of 10 fish was taken from each aquaria for individual length and weight data. On 30 March 1971, the number of fish per aquarium was reduced to 20 fish. These fish were individually marked with a cold brand utilizing liquid nitrogen and branding tools (Mighell, 1969). Growth rates of these individuals were followed during subsequent measuring periods. Analysis of co-variance was used to determine possible lead related growth differences.

Sacrificing for hematological investigations

The hard and soft water fish were sacrificed periodically to adjust number of fish per aquarium and as crowding was observed. Fish were sacrificed at the 4th, 6th, 8th, 11th, 15th and 19th months of the experiment. The number of fish sacrificed per sampling period averaged about 10 and depended somewhat on the number of mortalities that may have occurred. These fish were randomly selected, anesthetized with 0.2 g/gal MS-222, weighed and measured.

A variety of techniques have been described in the literature for collecting blood from various size fish (McCay, 1929; Root, 1931; Dawson, 1935; Field, Elvehjem and Juday, 1943; Schiffman, 1959; Hesser, 1960; Hunn, Schoettger and Whealdon, 1968; and Klontz *et al.*, unpublished). Of these, cardiac puncture and puncture of the dorsal aorta are difficult for fish under 6 inches in length. For small fish, severance of the caudal peduncle has proven to have the greatest utility.

After anesthetization in MS-222, the fish was wrapped in a large absorbent towel to remove excess water and allow an adequate means for holding the fish. A stainless steel scalpel was used to sever the caudal peduncle. Chemists at Colorado State University have found metal contamination in tissues after using scalpels that were not stainless steel (R. K. Skogerboe, personal communication). The caudal peduncle was severed approximately midway between the adipose fin and the caudal fin. The flowing blood was collected in capillary tubes that were filled with heparin solution (1 ml = 1000 USP units) and blown empty for removal of excess heparin. Regular heparinized capillary tubes have proven inadequate in preventing clotting of rainbow trout blood.

Heparin was selected because: Wintrobe (1934) preferred heparin as an anticoagulant. Hesser (1960) found heparin the most satisfactory with crenation and/or lysis of the red blood cells minimal. He also stated that EDTA (ethylenediamine tetracetic acid) salts, ammonium and potassium oxalate and citrates gave fair results. Some crenation occurs with the use of oxalates. Other anticoagulants have been used in fish hematology. McCay (1929) used mineral oil on needles and syringes and oxalates and citrates in test tubes. Duthie (1939) coated containers with wax. Field *et al.* (1943) used 0.1 *M* sodium oxalate or heparin in a syringe.

Hematological methods

Three capillary tubes of heparinized blood were collected from each fish. Blood from one tube was used in making a hemoglobin determination. The second capillary tube was used in making hematocrit and plasma protein determinations and the third was kept as a spare. The cyanmethemoglobin method of determining hemoglobin (Seiverd, 1964) is preferred by this laboratory and employs a Bausch and Lomb Spectronic 20. Klontz *et al.*

(unpublished) has also recommended this method for standard use with rainbow trout. Hesser (1960) preferred the acid hematin method to the cyanmethemoglobin method because of the formation of a proteinecious gel in the latter which he felt gives inaccurate readings by obtaining solutions of varied densities.

It is believed that the cyanmethemoglobin method far outweighs other disadvantages inherent in the acid hematin method, those of time and temperature variations, stability of the hematin color and the effects of lipids and plasma protein of color development. It was found that by swirling the cuvette in the cyanmethemoglobin method that the gelatinous precipitate will normally float to the surface and will not inhibit the passage of light in a colormetric determination. The gelatinous precipitate can then readily be removed from the sample by use of a wooden applicator.

Hematocrits were determined by the micro-hematocrit method (Hesser, 1960; Seiverd, 1964). The capillary tube, following 10-min centrifugation using an International Model MB Micro Capillary Centrifuge, was broken above the point of separation between the cell pack and plasma fractions. Plasma protein levels were determined using American Optical Company T. S. Meter, Model 10401 (a Goldberg Refractometer). Results are reported in g/100 ml. The plasma was placed onto the refractometer and the plasma protein concentration read off the protein scale.

Soft Water Reproduction Bioassay

Fish and toxicant system

The soft water reproduction bioassay was initiated on 21 August 1970 with a 2-year-old rainbow trout obtained from the Federal Genetic Research Hatchery at Beulah, Wyoming. Two-year-old fish were selected for spawning as 3-year-olds to alleviate problems associated with variable reproductive maturity existing with 2-year-old rainbow females. Preliminary observations indicated that four fish were the maximum number per 325-liter aquarium permitting free movement and the maintenance of good environmental conditions. The fish used in this test were selected on the basis of compatibility and adaptation to aquarium conditions. Two males (averaging 720 g) and two females (averaging 892 g) were placed in each of the six test aquaria. A 33.3% dilution ratio with a high nominal concentration of 30 µg/liter was also used in this experiment.

Feed and feeding rate

Fish used in the reproduction bioassay were fed a maintenance ration of Rangen 7/32" brood pellets at 1% of body weight per day. A weekly supplement of beef liver was also fed.

Spawning and hatching

Plastic pans (30 cm x 25 cm x 15 cm) were filled to an approximate depth of 8 cm (6 kg) with gravel (15-30 mm diameter) and placed in the reproduction bioassay aquaria in an attempt to obtain natural spawning. With attempts unsuccessful in obtaining natural spawning, the fish were anesthetized with MS-222 and spawned artificially. Fertilized eggs were placed on nylon net egg trays in 35-liter aquaria of the acute bioassay system. The eggs of each female were hatched in separate aquaria. A 50% diluter with a nominal high concentration of 100 μg/liter lead supplied the toxicant.

SOFT WATER F_1 Generation Reproduction Bioassay

Fish and toxicant system

At an age of 1 month, 250 successfully hatched fry from each concentration were transferred from hatching aquaria to the large 325-liter aquaria. As with the hatching aquaria system, a 50% diluter with a nominal high concentration of 100 μg/liter supplied the toxicant. The experiment was terminated 15 December 1971.

Feed and feeding rates

The feeding rates for the F_1 reproduction bioassay were adjusted at 2-week intervals and based on the total weight of fish per aquarium. Rates concur with the modified Cortland feeding chart. A weekly beef liver supplement is also fed. At the age of 6 months, the number of fish per aquarium was reduced to 30. Subsequent to this time, the feeding rate was adjusted daily based on a computerized growth rate projection and adjusted every 2 weeks from current weight data.

Growth measurements

Growth measurements of the F_1 reproduction fish were collected every 2 weeks. Initially this consisted of a count and total weight measurement of fish per aquarium. At an age of 6 months the fish were cropped, leaving a total of 30 fish per aquarium. Dr. David Bowden, statistician with Colorado State University, stressed the need for a large sample size to reduce within sample variation where individually marked fish did not exist. The total length in millimeters of individual fish sacrificed was determined and will be used as the index for measuring growth differences between lead concentrations.

The growth bioassay experiments indicated a possible effect of aquarium space on growth where the number and size of fish varied from one concentration to another. Utilizing a proportional space-weight relationship, aquaria were divided with a plexiglass divider. For example, if the control aquarium has 1000 g of fish and the high concentration has 500 g of fish, the available space in the control aquarium would be considered at 100% and the high concentration aquarium with one-half the weight of fish would be divided in half. Therefore, the available space per gram of fish would be the same for both aquaria.

ACUTE BIOASSAY METHODS

Hard Water Acute Bioassays

Flow-through bioassay

One hard water acute bioassay was performed. A proportional diluter calibrated for a 25% dilution ratio delivered a nominal high concentration of 10 mg/liter. Ten fish were placed in each of six 35-liter aquaria. The test rainbows were pre-acclimated to hard water for at least 30 days prior to being transferred to the acute bioassay aquaria. Total lead concentrations in each aquaria were analyzed from nonacidified samples by atomic absorption. Individual fish length and weight data were collected.

Static bioassays

Three static well water bioassays were conducted with 10 rainbow trout per concentration using 35-liter aquaria. The aquaria were aerated with a large airstone to provide mixing and supply dissolved oxygen. Routine water chemistries consisting of hardness, alkalinity, pH, temperature and dissolved oxygen were performed. Lead concentrations were analyzed from acidified samples by atomic absorption spectrophotometry, and by pulse polarography in static bioassay #3. Individual fish length and weight data was collected.

The first static bioassay was used to determine the approximate toxicity of lead in hard water. The nominal lead concentrations were 50, 100, 300, 500, 600 and 1000 mg/liter. The second static bioassay was used to "pinpoint" a TL_{50} concentration because of the inability of running a flow-through bioassay which would have exceeded the solubility of lead in the diluter stock solution. The nominal lead concentrations were 500, 520, 540, 560 and 580 mg/liter. The third static bioassay was run so that pulse polarographic, free lead, data might be obtained. The determination of TL_{50} concentrations were made by log-probit analysis (Sprague, 1969).

Soft Water Acute Bioassays

The initial soft water flow through acute bioassays employed a 25% dilution ratio and failed to produce mortality differences between high and low lead concentrations.

Subsequent soft water acute bioassays were performed using a 50% dilution ratio. Each of the test concentrations were duplicated in two 35-liter aquaria by dividing flows from the respective diluter reservoirs. Routine water chemistries and lead analyses were made on each acute test. Chemical analyses were made for alkalinity, hardness, pH, dissolved oxygen, temperature and conductivity. Fish were fed at an appropriate feed rate in the soft water acute bioassays since the time of exposure exceeded 10 days. The TL_{50} concentration was determined by a computerized log-probit analysis (Daum, 1969; and Daum and Killcreas, 1966).

Section V

RESULTS

CHRONIC BIOASSAY RESULTS

Hard Water Growth Bioassay

Introduction

The hard water growth bioassay was initiated 14 May 1970 using 70 fingerling rainbow trout averaging 83.3 mm in length and 6.1 g in weight. The purpose of this experiment was to determine lead-attributed mortality, growth or other effects that could be used to calculate an application factor, and to test the application factor concept as it applies to two different water qualities. The experiment was terminated 15 December 1971 after 19 months of chronic lead exposure.

Water analysis

Detailed chemical analyses were made on the hard water source (Table 1). Of particular interest is a mean hardness of 353.5 mg/liter a methyl orange alkalinity of 243.1 mg/liter, and a mean pH of 8.02. These values were determined from the control aquarium while occupied by fish. Metal analysis on the water source was performed by atomic absorption spectrophotometry except for the base lead levels which were determined by pulse polarography. Metal analyses of particular interest are lead, zinc and copper which in hard water were found to be 0.003, 0.03 and 0.005 mg/liter respectively.

Table 2 gives the chemical results from weekly water analyses over duration of experiment for dissolved oxygen, pH, conductivity, alkalinity, hardness and temperature. The mean, standard deviation and range of a particular analysis are reported. The dissolved oxygen in the six experimental aquaria was 7.015 $\pm$ 0.405 mg/liter. An analysis of variance accompanied with a least significant difference test (Sokal and Rohlf, 1969) showed that the dissolved oxygen levels in aquarium C (control) were significantly different from the other aquaria (Table 2); however, this was not considered biologically significant. The higher dissolved oxygen levels of the control aquarium probably result from fewer number of fish in this aquarium due to a large number of mortalities that occurred early in the experiment. Statistical analysis on pH, conductivity, alkalinity, hardness and temperature revealed no significant difference

Table 1. Mean chemical analysis of hard and soft water supplies (concentrations in mg/liter).

Analysis	Hard water	Soft water
Alkalinity M.O. (as $CaCO_3$)	243.1	26.4
Alkalinity phth (as $CaCO_3$)	0.0	0.0
Hardness (EDTA)	353.5	27.2
Chloride	23.65	3.45
Nitrate-nitrite nitrogen	25.52	0.95
Dissolved silica (molybdate-reactive)	16.4	8.7
Sulfate	103.2	2.4
TDS (total dissolved solids)	584	62
Specific conductance (micromhos/cm)	1456	170
Dissolved oxygen	7.42	7.04
pH	8.02	6.88
Cadmium	0.00	0.00
Calcium	75.9	10.8
Chromium	0.00	0.00
Copper	0.005	0.005
Iron	0.05	0.05
Lead	0.003	0.003
Magnesium	29.68	2.71
Manganese	0.00	0.00
Molybdenum	0.0	0.0
Nickel	0.00	0.00
Potassium	1.20	1.35
Silver	0.00	0.00
Sodium	14.72	9.40
Zinc	0.03	0.02

Table 2. Average water quality for hard water growth bioassay (May 1970-December 1971).

Tank	H-1	H-2	H-3	H-4	H-5	H-Control
Dissolved oxygen (mg/1	7.01	6.61	6.57	7.02	6.98	7.42
Standard Deviation	0.92	1.01	1.09	0.90	1.05	0.80
Range	4.2-9.2	3.0-8.3	4.0-10.4	4.8-9.0	3.6-11.5	4.6-8.8
pH	7.86	7.88	7.88	7.92	7.92	8.02
Standard deviation	0.17	0.19	0.18	0.17	0.19	0.15
Range	7.49-8.27	7.48-8.26	7.46-8.28	7.59-8.20	7.66-8.29	7.64-8.25
Conductivity	1450.2	1476.6	1463.9	1448.0	1450.0	1456.5
Standard deviation	114.87	107.97	116.06	117.11	118.96	112.07
Range	1200-1800	1225-1900	1200-1900	1125-1650	1150-1800	1200-1750
Alkalinity mg/1 ($CaCO_3$)	242.3	242.7	243.0	242.6	242.8	243.1
Standard deviation	17.20	17.36	17.78	17.26	17.42	18.21
Range	210-290	210-290	210-290	210-290	210-290	210-298
Hardness mg/1 ($CaCO_3$)	353.5	352.1	353.2	353.6	353.4	353.5
Standard deviation	24.98	26.42	24.90	24.52	24.78	24.70
Range	276-400	276-400	276-400	276-400	276-400	276-400
Temperature (^{o}C)	13.84	14.96	14.90	13.99	14.03	15.12
Standard deviation	3.41	2.72	2.68	3.29	3.21	2.76
Range	5.0-20.0	9.7-20.5	9.7-21.0	7.5-19.5	7.5-20.0	9.0-21.0

between the test aquaria. Chemical results for the six hard water aquaria were pH (7.94 ± 0.08), conductivity (1462.3 ± 1.43), M.O. alkalinity (242.7 ± 0.4 mg/liter), hardness (352.85 ± 0.75 mg/liter) and temperature (14.48 ± 0.64°C).

Lead analysis

Average values in the six hard water aquaria for lead concentrations determined by pulse polarography and atomic absorption are given in Table 3. Equilibrium calculations show that the total solubility of lead in hard water at a pH of 8.0 is 30 μg/liter (Figure 12 in appendices). However, as seen from Table 3b, pulse polarographic analysis for free lead, aquaria #1, #2 and #3 exceeds the calculated solubility. The difference between analyzed concentrations and the calculated solubility are not surprising, considering the uncertainties of the equilibrium calculations because of the inaccuracies obtained in defining the experimental conditions from which the calculations were made. The nominal or added concentrations of lead are much higher than the calculated solubility (Table 3). Therefore, only a fraction of the total lead added would exist as soluble species in this water, with the remaining lead existing as colloidal and precipitated forms.

Comparing pulse polarography and atomic absorption results, one can see that the lead concentrations found by atomic absorption are much higher than the corresponding values obtained by pulse polarography (Table 3). These results are qualitatively those to be expected, because lead (II) precipitates are generally slow to form and aggregate. This means that substantial amounts of colloidal material can be present, suspended in the samples. The atomic absorption analysis method will analyze such material, whereas the pulse polarography method will not. Therefore, one can roughly say that analysis of water samples by atomic absorption gives total lead, consisting of dissociated and suspended forms but not that which has precipitated to the bottom of an aquarium. Conversely, pulse polarography will measure only that lead which is dissociated or free in solution. (See Appendix C for further discussion of problems associated with analyzing lead by atomic absorption).

Analysis of variance and least significant difference tests at a 95% confidence interval on the hard water lead data revealed a significant difference between the atomic absorption analyzed lead concentrations for H-1 (2.03 mg/liter), H-2 (0.78 mg/liter) and H-3 (0.37 mg/liter). However, nonsignificance was found between H-3, H-4 (0.20 mg/liter) and H-5 (0.10 mg/liter) and is believed to be the result of analytical problems associated with analysis of these lower lead levels by atomic absorption and the inability of knowing what lead species are present in any particular sample. For this reason, the computed concentrations obtained from the analyzed H-1 concentration of 2.03 mg/liter lead might be considered to be the most reliable. However, any atomic absorption analysis of lead in hard water gives relatively meaningless results because of the various types of complexes, suspended or precipitated, which may or not be present in

Table 3. Hard water lead analytical results (concentrations in mg/liter).

	Aquarium #					
	H-1	H-2	H-3	H-4	H-5	H-C
a. Hard water growth bioassay analyzed by atomic absorption						
Nominal concentration*	3.24	1.08	0.36	0.12	0.04	0.00
Analyzed concentration	2.03	0.78	0.37	0.20	0.10	0.00
Standard deviation	0.76	0.26	0.13	0.07	0.03	
Range	0.5-3.4	0.3-1.2	0.1-06	0.1-03	0.05-0.20	
Computed concentration from high analyzed concentration	2.03	0.68**	0.23**	0.07**	0.03**	--
b. Hard water growth bioassay analyzed by pulse polarography						
Nominal concentration	3.24	1.08	0.36	0.12	0.04	0.0
Analyzed concentration*	.064	.044	.032	.018	.011	.003
Standard deviation	.008	.031	.005	.004	.002	.001
Range	.005-.087	.032-.090	.021-.043	.010-.027	.010-.015	.002-.004
Computed concentration from high analyzed concentration	.064	.021**	.007**	.002**	.001**	--

* Values used in report.

** Computed from analyzed high concentration.

proportion to their presence in an aquarium at the time a sample is taken. Therefore, stating that atomic absorption analysis represents total lead is not completely correct because of the absence of precipitated lead species which may not be collected in a grab sample.

Because of problems associated with hard water total lead analyses by atomic absorption spectrophotometry, the nominal concentrations, that is, the amount of lead added to any particular aquarium, are considered to be the most reliable. The actual amount of lead added to each aquarium can readily be determined by knowing the concentration of the stock solution and the dilution ratio of the proportional diluter. The nominal concentrations, in mg/liter, are: H-1 (3.24), H-2 (1.08), H-3 (0.36), H-4 (0.12), H-5 (0.04) and H-control (0.00). The analyzed lead concentrations determined by pulse polarography represent free lead values. These are, in mg/liter: H-1 (.064), H-2 (.044), H-3 (.032), H-4 (.018) and H-5 (.011). The pulse polarographic concentration for lead in the hard water control aquarium, H-C (0.003 mg/liter), will be used as the base lead level for hard water.

Growth results

The growth data for the fish in H-C (the hard water control aquaria) was found to be statistically larger than the remaining five aquaria. This growth difference is attributed to drastic reduction in the total number of fish in the aquaria 4 months after initiation of the experiment. An error in the feeding rate for these fish also contributed to their greater growth. No statistical growth difference was found for the remaining five aquaria, H-1 (3.24 mg/liter Pb) through H-5 (0.04 mg/liter Pb). Condition coefficients, $K = 10^5$ weight/(length)^3, were determined and found to be nonsignificant between the six test aquaria.

Occurrence of physical abnormalities

Black tails were first noted in the high hard water concentration, aquarium H-1 (3.24 mg/liter Pb), 6 months after the initiation of the experiment. One month later in December of 1970, some fish started exhibiting eroded caudal fins and spinal curvatures. By March of 1971, the "blacktail" effect was observed to be 100% in H-1, 80% in H-2 (1.08 mg/liter Pb), and 1% in H-3 (0.36 mg/liter Pb). Aquaria H-4 (0.12 mg/liter Pb), H-5 (0.04 mg/liter Pb) and the control H-C (0.003 mg/liter Pb), did not demonstrate this effect. Upon terminating the experiment in December of 1971, the percentage of black tails, spinal curvatures (lordoscoliosis), and eroded caudal fins was determined for the final 10 fish removed from each of the six aquaria (Table 4). Black tails were not observed in the lower level concentration aquaria (H-4, H-5 and control) at any time during the experiment. From these data the maximum acceptable toxicant concentration ("MATC") in hard water was found to occur between the nominal concentration of 0.12 mg/liter (aquarium H-4)

and 0.36 mg/liter (aquarium H-3). Table 4 gives the percent of affected fish for both free and total lead concentrations.

Table 4. Percent of fish affected by physical abnormalities in the final sampling of the hard water growth bioassay (based on 10 fish).

Abnormality	H-1	H-2	H-3	H-4	H-5	H-C
Black tail	100	90	70	0	0	0
Lordoscoliosis	100	60	10	0	0	0
Eroded caudal fin	30	20	10	0	0	0
Total Pb (mg/liter)	3.24	1.08	0.36	0.12	0.04	0.00
Free Pb (mg/liter)	.064	.044	.032	.018	.011	.003

Discussion relating the effects of lead to various physical abnormalities

The blacktail phenomenon entails a process whereby the entire caudal region just posterior to the dorsal fin is blackened (Figures 1, 5 and 6). Haider (1964) created caudal region blackening by injecting lead into the top of the ventral fins. This occurs at or posterior to the first caudal vertebra (or 32nd vertebra). Black tails preceded occurrences of spinal curvature by at least a month and they were not found to begin simultaneously--at least not by observing the exterior morphology of the fish. Hoffman in his work with whirling disease attributed black tails to pressure exerted on the sympathetic nerves which control the caudal pigment cells (Hoffman, Dunbar and Bradford, 1962). The pressure arises from a spinal curvature in the region of the 26th vertebra from which the sympathetic nerves originate. In the lead work it is possible that vertebral cartilage damage, with a resulting effect on the sympathetic nerves, occurred prior to an observable flexure in the spinal column, and that the blacktail phenomenon arises subsequent to vertebral damage.

The spinal curvatures found in the fish exposed to lead were of two types: scoliosis (bilateral spinal flexures - see Figures 2 and 4, and/or lordosis (dorso-ventral spinal flexures - see Figures 1, 3, 5, 6, 7, and 8).

The lead-exposed fish which exhibited spinal curvatures usually demonstrated both types, in which case the curvature would be described as lordoscoliosis. The spinal curvature usually started at about the 26th vertebra and extended posteriorly. Figures 9 and 10 are X-rays of a normal fish.

Accompanying the two conditions of black tails and lordoscoliosis, 40% of the affected fish have also exhibited extensive erosion of the caudal fin with an associated hemorrhagic area at its base, possible again as a result of neural damage, associated bacterial infection, and/or biochemical blockage (Figures 1 and 3). Also observed and not previously reported in association with lordoscoliosis spinal abnormalities is the total or nearly complete paralysis of the entire caudal region and flexured portion of the fish. A progressive muscular atrophy of the caudal region was observed with or subsequent to paralysis. This is a chronic disorder marked by progressive wasting away of the muscles with paralysis.

Observations of the affected lead-exposed fish revealed that swimming was accomplished by motion or bending anterior to the caudal and flexed portions of the fish. The affected fish were normally lethargic, lying on the aquarium bottom, but on occasion would dart around the aquarium. They exhibited difficulty in maintaining position or equilibrium and would swim tilted to one side. Quivers or trembling movements were observed periodically as a radiating wave through the caudal region. Haider (1964) also reported symptoms of lying on bottom, darting movements, tilted swimming and wave-type muscular movements over the body.

It would appear very doubtful that the fish exhibiting pronounced lordoscoliosis would be able to spawn under natural stream conditions because of the paralysis and flexure of nearly half of the fish. One gravid female existed in the H-1 aquarium upon termination of the experiment 15 December 1971. This fish was artificially spawned and the eggs fertilized by milt from two ripe males of the H-1 aquarium. The fertilized eggs, which had very pale coloration, were placed in a hatching aquarium with the flowing water temperature adjusted to 11°C. Of the 1000 eggs collected, 100% mortality occurred within 14 days. A definite statement cannot be made regarding possible inhibition of viable egg reproduction at this concentration of 3.24 mg/liter lead, because of a sample size of one fish.

Comparison of similar disease related abnormalities with those found in the lead experiments

As previously stated, Hoffman reported similar occurrences of black tails and spinal curvatures as being symptomatic of whirling disease (Hoffman, Dunbar and Bradford, 1962). The lead-exposed fish were examined by our fish pathologist for the possible parasitic infection of *Myxosome cerebralis*, the protozoan that causes whirling disease. The fish were found to be free of the protozoan.

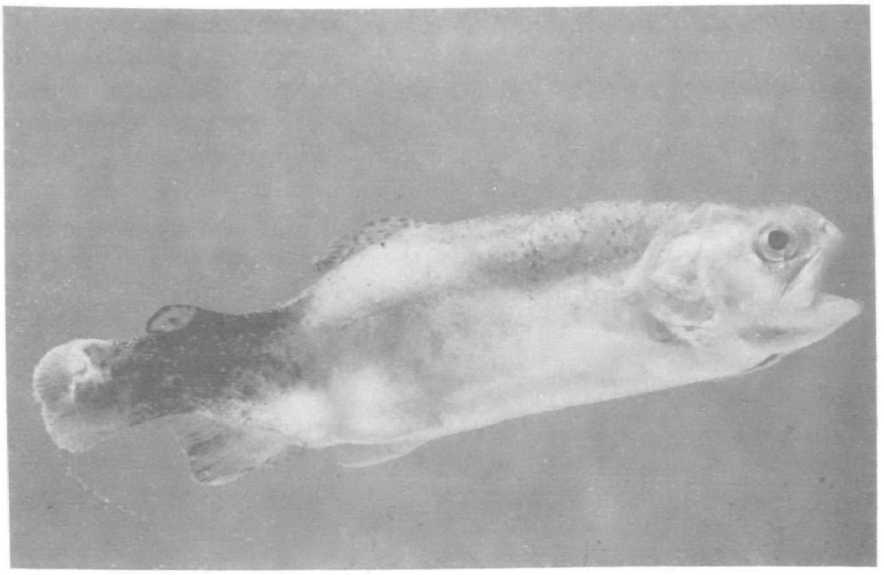


Figure 1. Fish no. R-0, H-1 (.064 mg/liter Pb) - lateral view. Note severe caudal fin erosion and cold brand.

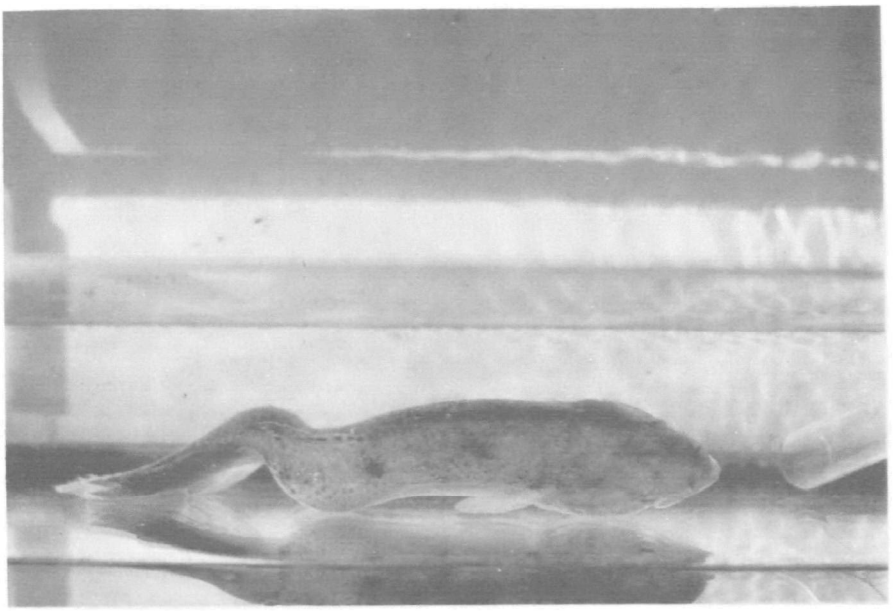


Figure 2. Fish no. R-0, dorsal view. Note extreme scoliosis.

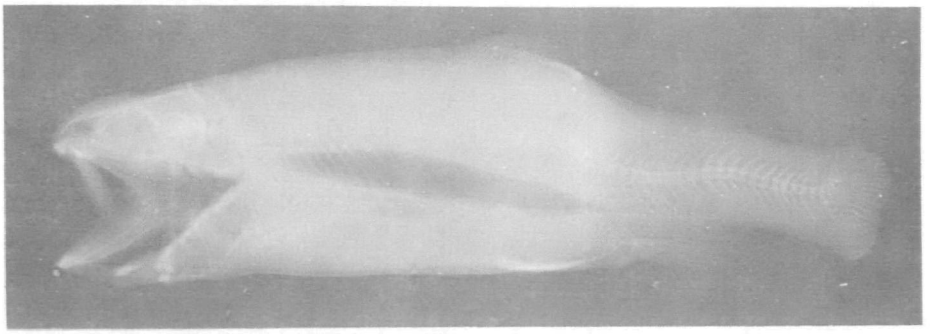


Figure 3. Fish no. R-0, X-ray - lateral view. Note spinal column flexures and caudal fin erosion.

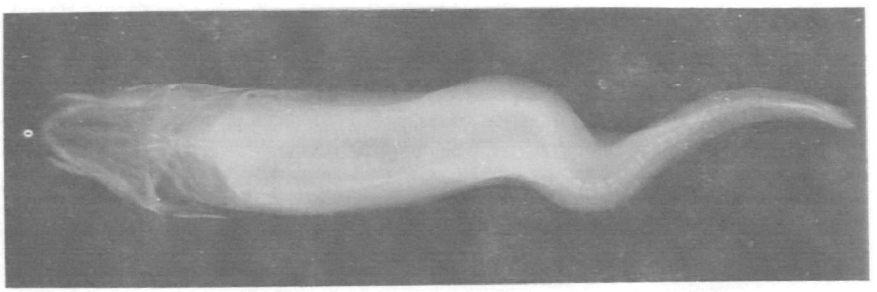


Figure 4. Fish no. R-0, X-ray - ventral view. Note extreme scoliosis of spinal column.

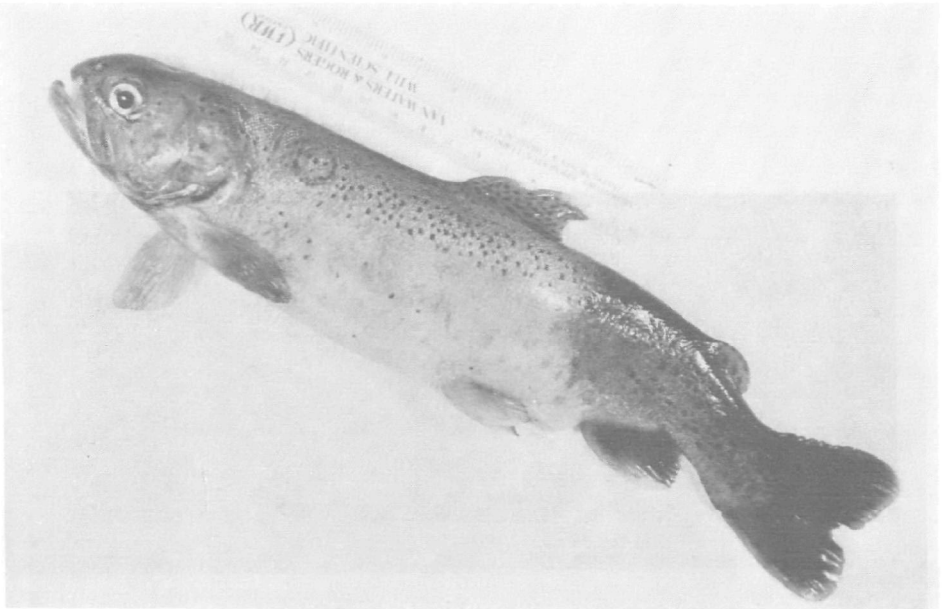


Figure 5. Fish no. L-10, H-1 (.064 mg/liter Pb) - lateral view. Note ventral lordosis and cold brand.

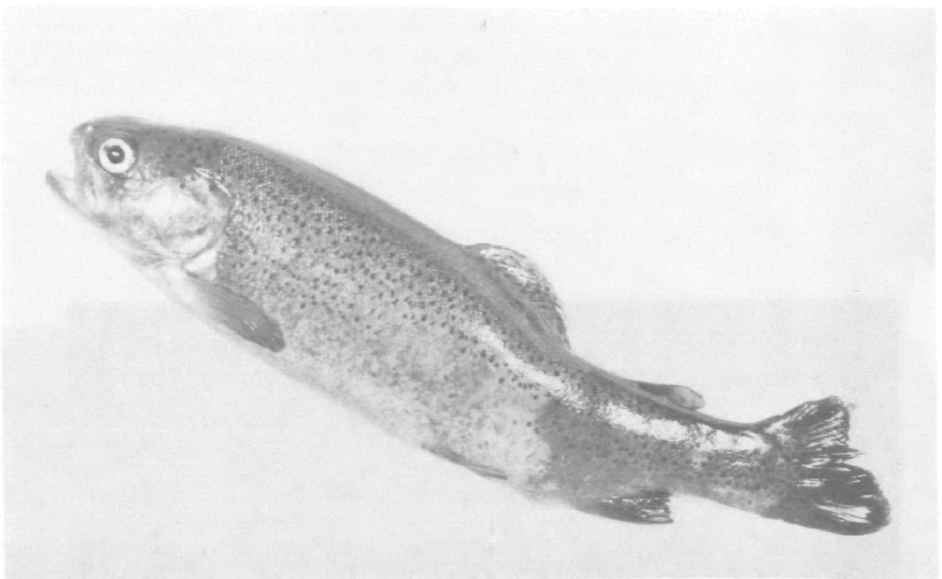


Figure 6. Fish no. R-1, H-1 (.064 mg/liter Pb) - lateral view. Note dorsal lordosis.

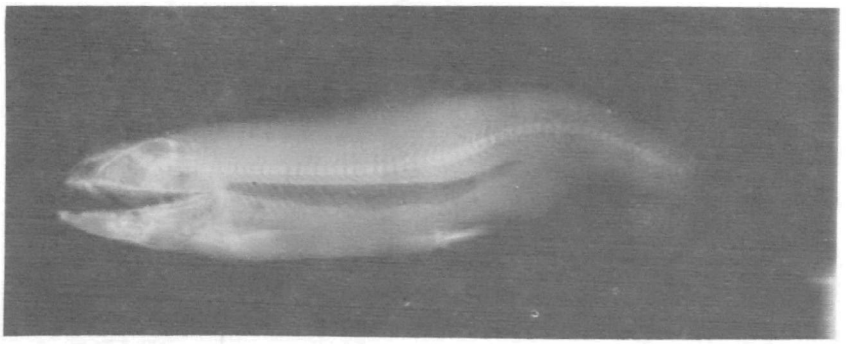


Figure 7. Fish no. L-10, X-ray - lateral view. Note ventral lordosis of spinal column.

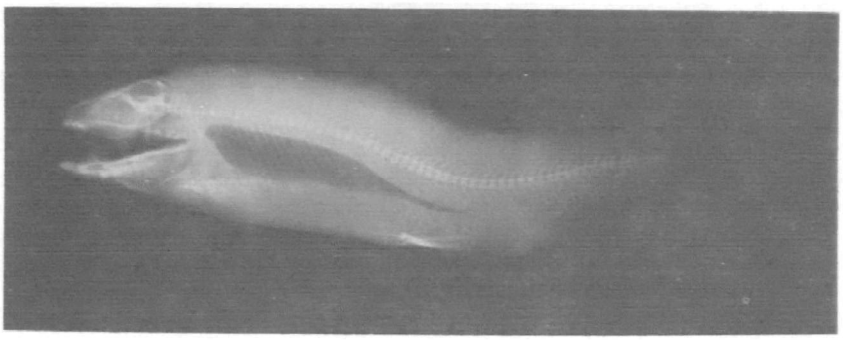


Figure 8. Fish no. R-1, X-ray - lateral view. Note severe dorsal lordosis.

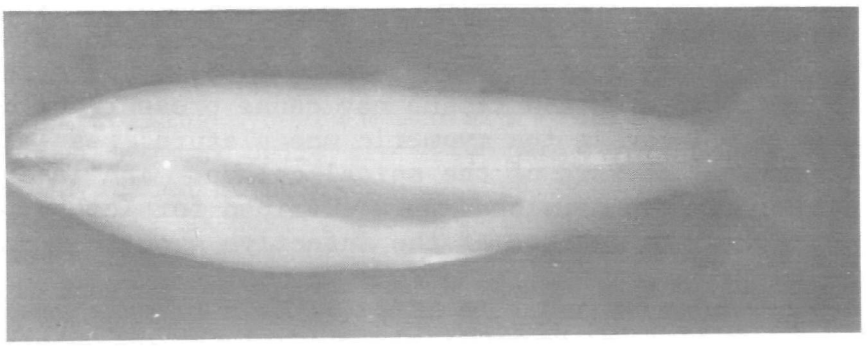


Figure 9. Control fish, X-ray - lateral view. Note no flexing of spinal column.

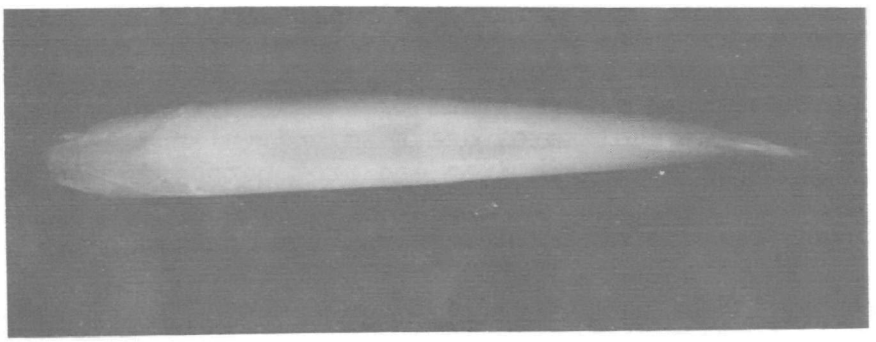


Figure 10. Control fish, X-ray - ventral view. Note no flexing of spinal column.

Reichenback-Klinke and Elkan have reported that abnormal spinal curvatures have been seen in: (a) hormonal imbalance; (b) faulty genetic factors; and (c) chronic parasitic infections with Icthyosporidium hoferi, mycobacteria and Acamthocephala (Reichenback-Klinke and Elkan, 1965). Icthyosporidium hoferi (Icthyophonus hoferi) is a fungal parasite found to infect trout and most other fish species (Reichenback-Klinke and Elkan, 1965). The organism has been found in skin lesions, gills, kidney, liver, spleen, intestine and brain tissues (Erickson, 1965). The effect of the organism on replacement of brain tissue may cause pressure or damage to the motor nerves which govern the myomeric musculature, resulting in a characteristic sigmoid flexure of the spinal column. The lead-exposed fish in which spinal curvatures developed were examined for Icthyosporidium spheres and were found to be free of the infective agent.

Nutritional deficiencies of ascorbic acid (vitamin C) have also been reported to cause acute scoliosis and/or lordosis in trout (Poston, 1967). Neuhaus and Halver reported a twisting in cartilage in gill filaments long before acute damage was observed in the spinal column, gill cover or eye support cartilage (Halver, 1970 and Neuhaus and Halver, 1969). Another nutritional deficiency was reported by Shanks who found pronounced scoliosis in rainbow trout fed diets deficient in the amino acid tryptophan (Shanks, Gahimer and Halver, 1962). Halver (1970) reported profound scoliosis and lordosis occurring within 4 weeks in rainbow trout in which tryptophan was deleted from the diet.

A question might be raised regarding a possible inhibitory effect of lead on the biochemical metabolism of vitamin C and tryptophan. de Bruin, 1971, reported (on research performed by Gontea and associates with guinea pigs) that lead decreases the normal vitamin C content of the adrenal gland (Gontea, 1964). It has also been suggested that lead interferes in some manner with the enzymatic degradation of tryptophan (Fati, 1961, as reported by de Bruin, 1971).

At the level of cellular metabolism, the best known adverse effect of lead is its inhibition of enzymes dependent upon the presence of free sulfhydryl (SH) groups for their activity (Chisolm, 1971). This effect is most clearly manifested in the biosynthesis of heme. Specifically, lead inhibits the enzymatic metabolism of delta-aminolevulinic acid (ALA) and the final formation of heme from iron and protoporphyrin (de Bruin, 1971). The enzyme that catalyzes ALA metabolism is ALA-dehydrase. This enzyme is extremely sensitive to the inhibitory effect of lead.

All cells synthesize their own heme-containing enzymes. Therefore, ALA-dehydrase is widely distributed in tissues. Millar and his colleagues (as reported by Chisolm, 1971) found that ALA-dehydrase activity was inhibited in the brain tissue of heavily lead-poisoned laboratory rats at about the same rate as it is in the blood suggesting that the enzyme may be implicated in brain damage. The toxic effect of lead in the central nervous system is little understood. Two different mechanisms appear to be involved in lead-caused brain damage (encephalopathy). (1) Edema: the walls of the blood vessels are somehow affected so that the capillaries become too permeable, causing a swelling of the brain tissue. Severe

swelling within the enclosed cavity can cause a destruction of brain tissue. (2) Direct injury to nerve cells: it appears that certain brain cells may be directly injured, or their function inhibited, by lead (Chisolm,1971).

In conclusion, it would appear that lead might exert its effect on the occurrence of black tails and spinal curvatures through two possible chanels: (1) by a biochemical inhibition in metabolism, or (2) by some direct or indirect influence on the central nervous system whereby motor nerves controlling myomere musculature and sympathetic nerve control of caudal pigment cells are affected. However, nothing conclusively can be stated at this time regarding the mode by which lead exerts an effect. It is possible that both channels could operate simultaneously. The conditions (i.e., black tails, spinal curvatures and paralysis) are highly suggestive of possible neurological damage to fish chronically exposed to lead. Chisolm, 1971, reports that another result of chronic overexposure to lead is peripheral nerve disease, affecting primarily the motor nerves of the extremities. The tissue damage appears to be in the myelin sheath of the nerve fiber. Specifically, the mitochondria of the Schwann cells, which synthesize the sheath, seem to be affected. The paralysis of nerves occurs mainly as radialis and fibularis paralysis. Direct lead damage to the central nervous system in humans can give rise to delirium, coma, epilepsy and lead eclampsia (Haider, 1965).

Mortality results

Four mortalities, believed to be lead induced, occurred in H-1, the high concentration of hard water aquarium having a nominal lead concentration of 3.24 mg/liter. These mortalities occurred on 15 June 1971, 11 August 1971, 10 September 1971, and 12 October,1971. The fish exhibited black tails from the caudal fin to the dorsal fin, acute lordoscoliosis, and complete erosion of the caudal fin occurred in three of the mortalities. A hemorrhagic area at the base of the caudal fin occurred. Prior to death these fish exhibited similar behavior: (1) showed no interest in food (anorexia), (2) had a great deal of difficulty in maintaining equilibrium or otherwise holding their positions in the water, (3) moved horizontally through the water with the head at a steep downward incline, and (4) remained largely inactive lying on the aquarium bottom. This behavior would continue as long as a month before death. Other mortalities would have undoubtedly resulted had the experiment not been terminated. Upon terminating the experiment 15 December 1971, two of the six remaining fish demonstrated the stressed behavior just described.

No lead-attributed mortalities occurred in any of the remaining five aquaria. On 3 and 7 September 1970 the control aquarium (H-C) had two periods of severe stress from low dissolved oxygen due to malfunctions with the diluter. A total of six mortalities occurred on these two dates.

Subsequent to this, however, was a continuous die-off which continued until 28 September with a total mortality of 51 fish. Examination after death showed no gross abnormalities other than a slight erosion of the gill lamellae. The fish were also examined at the U.S. Bureau of Sport Fisheries and Wildlife Hatchery at Springville, Utah, and found to be free of IPN (infectious pancreatic necrosis) and IHN (infectious hematopoetic necrosis). It would appear that the stress due to low dissolved oxygen may have caused irreversible damage to the fish which resulted in an extended period of mortality. The five fish that remained in this aquarium were very healthy for the remainder of the experiment.

Hematological results

Fish from the hard water growth bioassay were sacrificed on seven occasions during the experiment. An attempt was made to sacrifice 10 individuals each sampling period, but this number varied somewhat because of mortality adjustments that were made to maintain an equal number of fish per aquarium. The control aquarium H-C was sampled on only the first and last sacrifices because of the large number of mortalities that occured during September of 1970. The fish sampling periods were: 14 August 1970, 15 October 1970, 16 December 1970, 25 March 1970, 8 July 1971, 11 November 1971, and termination of 15 December 1971.

Analysis of variance at a 95% confidence interval was performed on the hematological parameters investigated (hematocrit, hemoglobin, and plasma protein) for the first five sampling periods, 14 August 1970, through 8 July 1971. Analysis revealed no consistent, significant difference between the experimental aquaria attributable to lead. Analysis was limited to these five occasions because the necessity of deleting results obtained from precocious males which have high hematocrit and hemoglobin levels. Analysis could not be performed on data obtained during those periods in which lead-attributed mortalities occurred again because of the necessity to delete data obtained from reproductively mature fish. As would be expected, fish with eroded caudal fins showed significantly reduced hematocrit and hemoglobin levels and a drastic reduction in the plasma protein level. These conditions are indicative of post hemorrhagic anemia due to blood losses from the hemorrhaged area at the base of the caudal fin.

Soft Water Growth Bioassay

Introduction

The soft water growth bioassay was initiated 14 May 1970 with 70 hatchery-reared, fingerling rainbow trout averaging 83.3 mm and weighing 6.1 g. The purpose of the experiment was to determine a soft water application factor

for lead and to test the application factor concept regarding its use for different water quality types. The experiment was terminated after 19 months of chronic lead exposure.

Water analysis

Table 1 gives a detailed chemical analysis on the tap (soft) water source. Noteworthy analyses are hardness (27.2 mg/liter), M.O. alkalinity (26.4 mg/liter) and pH (6.88). These values were determined from the control soft water growth aquarium. Metal analysis of the source water gave levels for lead, zinc and copper of 0.003, 0.02 and 0.005 mg/liter respectively.

Chemical results from weekly water analysis of the experimental aquaria are given in Table 5. The mean, standard deviation and range for a particular analysis are reported. The range for mean value in the six test aquaria are: dissolved oxygen (6.94 $\pm$ 0.17 mg/liter), pH (6.875 $\pm$ 0.045), conductivity (176.2 $\pm$ 1.2), methyl orange alkalinity (26.25 $\pm$ 0.25 mg/liter), hardness (27.05 $\pm$ 0.15 mg/liter), and temperature (14.40 $\pm$ 0.46^{o}C). Analysis of variance on these parameters gave a nonsignificant F value between the six aquaria.

Lead analysis

Average lead concentrations determined by pulse polarography and atomic absorption are given in Table 6. Equilibrium calculations show that the total solubility of lead in soft water at a pH of 7.0 is approximately 500 µg/liter (Figure 13 in appendices). The nominal concentrations of lead in soft water are considerably lower than the corresponding calculated solubility (Table 6). Consequently, most of the lead added would exist in the soluble form. The pulse polarography results for lead in soft water are approximately the same as the nominal values (Table 6). These results are to be expected because of the higher calculated solubility (500 µg/liter) versus the amount actually added.

Table 5. Average water quality for soft water growth bioassay (May 1970 - December 1971).

Tank #	S-1	S-2	S-3	S-4	S-5	S-Control
Dissolved oxygen (mg/l)	7.11	7.11	6.77	6.82	6.97	7.04
Standard deviation	0.75	0.80	1.02	0.97	0.97	0.94
Range	4.6-8.5	5.2-8.5	3.6-8.4	4.0-8.4	4.8-8.4	4.2-8.4
pH	6.92	6.91	6.88	6.83	6.86	6.88
Standard deviation	0.26	0.28	0.27	0.24	0.28	0.29
Range	6.00-7.55	6.32-7.60	6.21-7.80	6.34-7.70	6.34-7.90	6.29-7.90
Conductivity	174.1	173.1	176.8	172.7	177.4	170.5
Standard deviation	31.77	35.90	33.00	33.83	30.80	30.08
Range	115-270	120-320	110-280	115-300	115-260	120-290
Alkalinity mg/l ($CaCO_3$)	26.0	26.3	26.4	26.5	26.5	26.4
Standard deviation	5.95	5.96	5.88	5.83	5.88	5.84
Range	10-42	10-42	10-42	10-42	10-42	10-42
Hardness mg/l ($CaCO_3$)	26.9	27.1	27.2	27.2	27.2	27.2
Standard deviation	5.41	5.23	5.15	5.20	5.22	5.26
Range	18-40	18-40	18-40	18-40	18-40	18-40
Temperature (^{o}C)	14.47	13.94	14.85	13.94	14.82	14.07
Standard deviation	2.78	3.26	2.97	3.29	2.99	3.23
Range	8.5-19.0	7.5-20.5	8.5-21.3	7.5-20.8	8.5-22.2	7.5-21.0

Table 6. Soft water growth bioassay--lead analytical results (concentrations in μg/liter).

	Aquarium #					
	S-1	S-2	S-3	S-4	S-5	S-C
Analyzed by pulse polarography						
Nominal concentration	30.0	20.0	13.3	8.9	5.9	0.0
Analyzed concentration*	25.6	19.5	12.2	8.3	5.4	3.1
Standard deviation	3.5	2.6	2.0	1.4	1.0	1.0
Range	20-32	12-24	10-17	6-10	4-7	2-4
Computed concentration from high analyzed concentration	25.6	17.0**	11.3**	7.6**	5.0**	--
Analyzed by atomic absorption**	52.5	35.0**	23.3**	15.5**	10.3**	--
Standard deviation	27.0					
Range	5-110					

* Values used in report
** Computed from analyzed high concentration

Atomic absorption analyses were performed on the highest concentration aquarium (S-1) and gave an average lead level of 52.5 μg/liter. Calculated values for the other aquaria were obtained by multiplying the analyzed high concentration by a factor of 66.6%, a value corresponding to the 33.3% dilution ratio set by the diluter (Mount and Brungs, 1967). These concentrations are considerably higher than both the nominal and pulse polarography results. Such an occurrence might result from exceeding the analytical capability of the procedure and equipment used. Another possible or accompanying explanation could be a resulting accumulation of complexed lead in excess of that added to the aquaria due to the adherence of various lead forms to particulate matter in the tanks. Aquarium cleanliness was a problem requiring bi-weekly scrubbing of aquarium walls and resulting in large amounts of particulate matter partially suspended in the water. Water was removed from an aquarium via an overflow pipe which tends to decant clearer water from the surface with a concomitant buildup of denser particulate matter below the surface. This material tends to remain in suspension because of a circulating action caused by the aeration and filtration systems. Removal of particulate matter by filtration was a slow process. Atomic absorption analysis of acidified water samples containing such material would undoubtedly reflect higher than nominal lead concentrations, whereas pulse polarography would not.

The pulse polarography results for analysis of lead in soft water are considered to be the most reliable. These results yielded the following, in μg/liter: S-1 (25.6), S-2 (19.5), S-3 (12.2), S-4 (8.3), S-5 (5.4) and S-C (3.1). Analysis of variance and least significant difference tests showed significant difference between all concentrations at a 95% confidence interval.

Growth results

The growth data collected from the soft water growth bioassay showed no statistical difference in growth between the six experimental aquaria at a 95% confidence interval. Coefficient of condition factors, $K - 10^5$ weight/$(\text{length})^3$, and analyses of variance were determined on fish in the six aquaria, and revealed no statistical difference in condition at the same confidence level.

Occurrence of physical abnormalities

Fish with black tails were observed in the two high concentrations, S-1 (25.6 μg/liter Pb) and S-2 (19.5 μg/liter Pb). However, no black tails were found in the final sample in December 1971, because of the random sacrificing of these individuals for other experimental purposes. No physical abnormalities were observed in S-3 (12.2 μg/liter Pb), S-4 8.3 μg/liter Pb), S-5 (5.4 μg/liter Pb) or the control aquarium (3.1 μg/liter Pb). From these data it would appear that the "MATC" for lead in soft water would exist somewhere around 12 μg/liter. Results from the F_1 generation reproduction bioassay gave a pronounced "MATC" for soft water. These will be discussed later.

Mortality results

No lead-attributed mortalities occurred in the soft water growth bioassay. Ten fish died in the control aquarium on 15 April 1971 due to low dissolved oxygen because of an accidental shut down of the aeration system. The mortalities were replaced with marked fish of the same size that had been held in similar water quality.

Hematological results

Fish from the soft water growth bioassay were sacrificed on 18 August 1970, 14 October 1970, 16 December 1970, 25 March 1971, 12 July 1971, 11 November, and 15 December 1971. An attempt was made to sample 10

fish from each aquarium at each sampling period. However, in order to obtain equal numbers of fish in each aquaria, it was necessary to adjust the number of fish sacrificed to compensate for fish losses. The hematological parameters tested were hematocrit, hemoglobin, and plasma protein. Analysis of variance at a 95% confidence interval revealed no consistent significant difference attributable to lead in the various experimental aquaria. As was the case in the hard water hematological findings, only that data collected between August 1970 through July 1971, five sampling periods, were compatible for a reliable statistical analysis because of the necessity of deleting data collected from precocious males. A nonsignificant effect of lead on the hematological parameters tested could be due to the low lead concentration of this experiment.

Water Temperatures in Soft and Hard Water Growth Bioassays

The hard (well) water with a year-round temperature of 15°C was cooled to the ambient soft (tap) water temperature except when soft water temperatures exceeded 16°C, in which case, the soft water was cooled to the hard water temperature. A t-statistic was used to analyze possible temperature difference between the hard and soft water growth bioassays and revealed no statistical difference.

Soft Water Reproduction Bioassay

Introduction

This experiment was complementary to the soft water growth bioassay with the purpose of determining the effect of lead on rainbow trout reproduction. The bioassay was initiated 21 August 1970 with 2-year-old fish, two males and two females, and terminated following spawning after 8 months of chronic lead exposure.

Water Analysis

Chemical results from weekly water analysis are reported in Table 7. These results from the six reproduction aquaria are dissolved oxygen (7.14 ± 0.21 mg/liter), pH (6.855 ± 0.055), conductivity (179.0 ± 3.1), M. O. alkalinity (29.85 ± 0.05 mg/liter), hardness (28.4 ± 0.3 mg/liter), and temperature 12.17 ± 0.58°C). Analysis of variance and a least significant difference test at a 95% confidence interval showed no significant difference in the chemical parameters tested.

Table 7. Average water quality for reproduction bioassay in soft water (August 1970 - May 1970).

Tank	R-1	R-2	R-3	R-4	R-5	R-Control
Dissolved oxygen (mg/l)	7.35	7.12	7.16	6.93	6.93	6.93
Standard deviation	0.96	0.92	1.09	1.06	0.89	0.90
Range	5.4-9.0	4.6-8.4	4.6-9.0	4.2-8.4	4.2-8.2	5.4-8.8
pH	6.91	6.90	6.83	6.83	6.85	6.80
Standard deviation	0.20	0.14	0.14	0.14	0.15	0.13
Range	6.45-7.33	6.62-7.18	6.48-7.09	6.55-7.20	6.62-7.20	6.53-7.08
Conductivity	175.9	182.1	178.3	181.5	181.1	179.7
Standard deviation	21.70	26.55	20.53	22.96	24.84	23.58
Range	145-230	135-265	135-230	135-235	135-250	140-250
Alkalinity mg/l ($CaCO_3$)	29.9	29.9	29.8	29.8	29.9	29.8
Standard deviation	4.92	4.92	5.03	5.03	4.92	5.03
Range	18-40	18-40	18-40	18-40	18-40	18-40
Hardness mg/l ($CaCO_3$)	28.1	28.7	28.6	28.6	28.6	28.7
Standard deviation	6.58	4.65	4.82	4.72	4.72	4.65
Range	22-40	26-40	20-40	20-40	20-40	20-40
Temperature (^{o}C)	11.59	12.72	11.64	12.75	12.62	11.68
Standard deviation	3.00	2.74	3.08	2.85	2.91	3.13
Range	7.2-18.7	9.0-19.8	7.2-18.8	9.2-20.5	7.2-20.2	7.2-19.0

Lead analysis

Lead results determined by pulse polarography and atomic absorption are reported in Table 8. As was previously found to occur in the soft water growth bioassay, the pulse polarography results closely approximated the nominal lead levels, whereas, atomic absorption findings computed from analysis of the high concentration were significantly higher than the nominal and pulse polarography lead levels. (Explanations for this were postulated under the soft water growth lead findings). Limited data and within group variance made statistical analysis impractical for pulse polarographic lead findings. For this reason the computed lead levels determined from the pulse polarographic analysis of the high concentration are considered to be the most reliable results. The lead concentrations in µg/liter are: R-1 (27.0), R-2 (18.0), R-3 (12.0), R-4 (8.0), R-5 (5.3) and R-C (2.6 - analyzed value.

Table 8. Soft water reproduction bioassay--lead analytical results (concentrations in µg/liter).

	Aquarium#					
	R-1	R-2	R-3	R-4	R-5	R-C
Analyzed by pulse polarography						
Nominal concentration	30.0	20.0	13.3	8.9	5.9	0.0
Analyzed concentration	27.0	17.5	10.4	7.7	6.0	2.6
Standard deviation	5.3	3.3	2.1	1.6	1.6	0.8
Range	21-34	10-23	8-15	6-10	4-8	2-4
Computed concentration from * high analyzed concentration	27.0	18.0**	12.0**	8.0**	5.3**	---
Analyzed by atomic absorption	58.5	39.0**	25.9**	17.3**	11.5**	---

* Values used in report
** Computed from analyzed high concentration

Occurrence of physical abnormalities

At termination the 3-year-old brood fish of the soft water reproduction bioassay did not demonstrate any of the physical abnormalities (i.e., black tails, lordoscoliosis or eroded caudal fins) as seen in some of the growth bioassay fish. With lead concentrations the same as in the soft water growth bioassay, which showed only a slight effect due to lead, the noneffect would probably be expected. One fish died in the soft water reproduction bioassay prior to spawning. This was a female in the lowest concentration aquaria (R-5 with 5.3 µg/liter Pb) that refused to eat and soon starved to death.

Spawning

Plastic pans (30 cm x 25 cm x 15 cm) were placed in each of the six test aquaria on 4 March 1970. Each pan contained 6 kg gravel (15-30 mm diameter) which yielded a depth of approximately 8 cm. Attempts at making a redd occurred in aquaria #2 (18.0 μg/liter Pb) and #5 (5.3 μg/liter Pb) and a few dead eggs were observed outside of the spawning pans in these aquaria on 24 March. Natural spawning did not occur in any of the aquaria, possibly as a result of competition or inadequate spawning pan size.

All fish were anesthetized in MS-222 on 31 March 1971 and attempts at artificial spawning were made. Females that were not gravid at this time were examined weekly until spawning occurred. Artificial spawning was completed 7 May 1971. All reproductive fish were numbered in accordance with the aquarium in which they resided. Thus, the fish in Aquarium #1 (highest lead concentration) became females #1a and #1b and males #1a and #1b. Fertilized eggs were placed on nylon net egg trays in 35-liter aquaria of the acute bioassay system. The hatching aquaria identification number coincided with the females from which the eggs were obtained with one aquarium used for hatching the eggs of a particular female. A 50% diluter with a nominal high concentration of 100 μg/liter supplied the toxicant. This concentration is considerably higher than that set for the parent generation (nominal high concentration of 30 μg/liter) in an attempt to obtain lead-caused mortalities in the high concentration.

Hatching and separation to large F_1 aquaria

Atomic absorption analysis of the high concentration for lead in the hatching aquaria (1a and 1b) was 101.8 μg/liter. Concentrations for the other aquaria were computed from the analyzed high concentration using the 50% diluter ratio. Successful hatching occurred in aquaria: #1a(101.8 μg/liter Pb), #2a(50.9 μg/liter Pb), #2b(50.9 μg/liter Pb), #3b(25.4 μg/liter Pb), #4a(12.7 μg/liter Pb), Ca(control, 3.1 μg/liter Pb, base level). Aquaria #1b, #3a, $4b, $5a, #5b, and Cb suffered 100% egg mortality. Surviving eggs took 29 to 30 days to hatch at a mean temperature of 12.66°C. The fry hatched from aquarium #2a exhibited 80% deformity. An explanation for the 100% egg mortalities at designated concentrations and for the deformed fry in aquarium #2a cannot be given. The extent to which lead-affected egg mortalities cannot be stated since mortalities occured in high and low concentrations alike. Weekly chemical data from the hatching aquaria showed a mean dissolved oxygen of 8.88 mg/liter (range 8.0-10.6) and a mean temperature of 12.66°C (range 10.0-14.0), which represents a mean saturation of 100%.

Viable fry were collected from at least one of the two reproduction females for all concentrations except aquarium #5 (parental lead concentration of 5.3 μg/liter or F_1 progeny lead concentration of 6.4 μg/liter). Two hundred and fifty fry were transferred from the hatching aquaria (#1a, #2b, #3b, #4a and Ca) to the large bioassay aquaria that their parents previously

occupied. With 100% egg mortality in aquaria #5a and #5b (6.4 μg/liter Pb), an additional 250 fry were transferred from the hatching aquarium #4a (12.7 μg/liter Pb) to the large chronic bioassay aquarium, #5. Tanks #1a and #4a completed hatching on 3 May and were separated on 3 June to the large aquaria (250 fish from hatching aquarium #1a to F_1 reproduction aquarium #1, and 500 fish from hatching aquarium #4a with 250 allocated to F_1 reproduction aquaria #4 and #5, respectively). Aquaria #2b, #3b and Ca/hatched on 31 May and 250 fish were transferred to the respective F_1 reproduction aquaria on 28 June 1971.

Use of 2-Year-Old Rainbow Trout as Spawning Stock to Study Toxicant Effects on Reproduction

The 2-year-old fish of the hard and soft water growth studies were to be used as brood fish. Examination of all remaining fish during the November 1971 sampling period, indicated a low percentage of reproductively mature females. Because of reproductive immaturity, the thought of using 2-year-old rainbow trout as brood fish was discontinued.

Data collected from the hard and soft water growth bioassays during the November and December sampling periods substantiated our decision to terminate the experiment. These fish were approximately 2 years old. Of the 51 fish remaining in the hard water growth bioassay, 27 were females of which 29.6% or eight were gravid. The remainder would not have produced eggs until next year. Fifty-seven fish remained in the soft water growth bioassay. Of these, 36 were females of which only 7 or 19.4% were gravid. The 29 other females would not have produced eggs until next year. Both experiments had one aquarium (H-3 and S-3) in which there were three gravid females. Aquaria H-4, H-5 and S-C had no reproductively mature females. Obviously, the percentage of reproductively mature females as 2-year-old rainbow trout is too low for providing a reliable brood stock and valid statistical results for studying effects of toxicants on reproduction.

Soft Water F_1 Generation Reproduction Bioassay

Introduction

This experiment was conducted on the progeny obtained from the 3-year-old brood stock of the soft water reproduction bioassay. The experiment ran for approximately 7 months from the date of hatching to 15 December 1971, when it was terminated. Lead levels were increased above those of the parent generation in an attempt to obtain lead-caused mortalities in the higher concentration aquaria.

Water analysis

Weekly water analyses were made on the six test aquaria (Table 9). The mean, standard deviation and range of the various determinations are reported. The range for the mean values are: dissolved oxygen (7.29 ± 0.15 mg/liter), pH (6.87 ± 0.04), conductivity (148.6 ± 2.2), methyl orange alkalinity (23.0 ± 0.3 mg/liter), hardness (23.95 ± 0.15 mg/liter) and temperature (16.31 ± 0.51 °C). An analysis of variance and least significant difference tests at a 95% confidence interval revealed no statistical difference between the six experimental aquaria for the chemical parameters analyzed.

Lead analysis

The average lead concentration for the two high concentration aquaria was determined by atomic absorption spectrophotometry. Lead levels in the other aquaria, F_1#3 through F_1C, could not be reliably determined because of detection problems associated with atomic absorption analysis. Aquarium F_1#2 reflected a higher than nominal lead concentration (Table 10) and probably corresponds to a lead concentration at which analytical problems with atomic absorption become apparent. For this reason, lead concentrations for aquaria F_1#2 through F_1#5 were computed using the 50% dilution ratio set by the diluter. Therefore, the reported lead concentrations for F_1 generation reproduction bioassay are, in µg/liter: F_1#1 (95.2), F_1#2 (47.6), F_1#3 (23.8), F_1#4 (11.9), F_1#5 (6.0) and F_1C (3.1) (Table 10).

Table 10. Soft water F_1 generation reproduction bioassay--lead analytical results (concentrations in µg/liter).

	Aquarium #					
	F_1-1	F_1-2	F_1-3	F_1-4	F_1-5	F_1-C
Analyzed by atomic absorption						
Nominal concentration	100.0	50.0	25.0	12.5	6.2	0.0
Analyzed concentration	95.2	52.6				
Standard deviation	16.8	15.6				
Range	60-130	20-90				
Computed concentration from* high analyzed concentration	95.2	47.6**	23.8**	11.9**	6.0**	3.1***

* Values used in report
** Computed from analyzed high concentration
*** Base level of lead

Table 9. Average water quality for soft water F_1 generation reproduction bioassay May 1970-December 1971)

Tank #	F_1-1	F_1-2	F_1-3	F_1-4	F_1-5	F_1-Control
Dissolved oxygen (mg/l)	7.44	7.26	7.37	7.19	7.15	7.13
Standard deviation	0.73	0.96	0.76	0.61	0.87	1.04
Range	6.2-9.0	4.0-8.2	5.8-8.2	6.0-7.8	6.0-9.6	5.0-10.0
pH	6.91	6.87	6.85	6.89	6.87	6.83
Standard deviation	0.24	0.22	0.20	0.30	0.30	0.24
Range	6.40-7.26	6.40-7.24	6.46-7.14	6.10-7.25	6.05-7.16	6.25-7.18
Conductivity	146.4	147.8	146.9	147.9	150.8	147.9
Standard deviation	11.57	14.58	13.92	13.67	15.92	12.65
Range	123-165	120-170	120-170	120-170	120-175	123-170
Alkalinity mg/l ($CaCO_3$)	22.8	22.8	23.3	22.7	22.7	23.1
Standard deviation	5.70	5.66	5.63	5.70	5.70	5.82
Range	16-34	16-34	16-34	16-34	16-34	16-34
Hardness mg/l ($CaCO_3$)	24.0	24.1	24.1	24.1	24.1	23.8
Standard deviation	4.92	4.92	4.92	4.92	4.92	4.84
Range	18-34	18-34	18-34	18-34	18-34	18-34
Temperature (^oC)	15.85	16.63	15.80	16.80	16.82	16.10
Standard deviation	1.17	0.82	1.24	1.05	1.04	1.08
Range	12.8-17.5	15.0-18.0	12.4-18.0	14.2-18.5	13.9-18.0	12.8-19.0

Growth results

Six months after hatching, 30 fish from each aquaria were analyzed for possible growth differences. Total length in millimeters was selected as the parameter for measuring growth. Since the fish in aquaria F_1#1, F_1#4 and F_1#5 hatched 1 month prior to the hatching of fish in aquaria F_1#2, F_1#3 and F_1C (control), the growth measurements were taken 1 month apart (at 6 months from the two hatching dates). Aquarium F_1#1 (95.2 μg/liter Pb) had a mean length of 113.87 mm, F_1#2 (47.6 μg/liter Pb) 99.13 mm, F_1#3 (6.0 μg/liter Pb) 92.67 mm, and F_1C (3.1 μg/liter Pb) had a mean length of 90.08 mm. Analysis of variance and a least significant difference test at a 95% confidence interval revealed that there was a significant difference in growth. Fish from F_1#1 were found to be statistically larger than fish from all other aquaria. F_1#2 was different from aquaria F_1#1, F_1#3 and F_1C. No statistical difference existed in growth between F_1#3, F_1#4, F_1#5 and F_1C.

These differences are not thought to be lead caused. The egg sources for each aquarium came from individual females of the reproduction bioassay. Eggs that were obtained from these females were of different sizes. The eggs obtained from high concentration reproduction bioassay R-1 were larger than those collected from any of the other brood females. Egg size, associated with possible genetic differences of the individual brood stock, are thought to cause the growth differences observed in the fingerling progeny. Because of these differences and problems associated with handling such data and data collection of fish with different hatching dates, it was decided to terminate the experiment.

Occurrence of physical abnormalities

Black tails were first observed in the F_1 reproduction bioassay 1 1/2 months after hatching. Table 11 gives the total number of fish per aquarium, percent black tails, eroded caudal fins and spinal curvatures. Initially, 250 fry were placed in each of the six experimental aquaria. This experiment was terminated on 15 December 1971.

Table 11. Percent of fish affected by physical abnormalities in the F_1 generation reproduction bioassay at termination of experiment.

	F_1#1	F_1#2	F_1#3	F_1#4	F_1#5	F_1C
Number of fish	76	207	193	204	238	192
Abnormality:						
Black tail	100.0	91.3	42.0	2.0	0.0	0.0
Lordoscoliosis	3.9	0.0	0.0	0.0	0.0	0.0
Eroded caudal fin	7.9	1.4	0.5	0.0	0.0	0.0

From these data the maximum acceptable toxic concentration ("MATC") exists between the lead concentrations of F_1#4 (11.9 µg/liter) and F_1#5 (6.0 µg/liter). These concentrations are in close agreement with the apparent "MATC" value of 12.2 µg/liter lead obtained in the soft water growth bioassay. The 2.0% black tails reported for aquarium F_1#4 represents four fish. Therefore, the true "MATC" value for lead in soft water is probably very close to the 11.9 µg/liter lead concentration. As witnessed in the hard water growth bioassay, one might expect the occurrence of lordoscoliosis to become much more pronounced as these fish increase in size.

Mortality results

Initially, 250 fish were transferred to each of the test aquaria from the hatching tanks. Fish were placed in aquaria F_1#1 (95.2 µg/liter Pb), F_1#4 (11.9 µg/liter Pb) and F_1#5 (6.0 µg/liter Pb) on 3 June, 1971, 1 month after hatching. Aquaria F_1#2 (47.6 µ g/liter Pb), F_1#3 (23.8 µg/liter Pb) and F_1C (control with 3.1 µg/liter Pb) received fish on 28 June, 1971, 1 month following egg hatching.

The experiment was terminated 15 December 1971. No mortalities occurred in aquaria F_1#1, F_1#4 and F_1#5 during the last 25 days of this period. Therefore, the mortality data presented in Table 12 are indicative of total mortality figures with equal time exposure to lead for all aquaria. Mortalities of 66.8% in aquarium F_1#1 clearly show a lead-caused effect. Mortalities in the lower concentrations are probably not attributable to lead. Table 12 gives the percent of fish unaccounted for. Cannibalism was observed, and the number of missing fish probably reflect the extent to which this occurred in each aquaria.

Table 12. Mortality results for F_1 generation reproduction bioassay based on initial 250 fish per aquaria.

	F_1#1	F_1#2	F_1#3	F_1#4	F_1#5	F_1C
Total no. fish accounted for	229	238	200	208	241	218
No. mortalities	153	29	7	4	3	26
% mortality	66.8	12.2	3.5	1.9	1.2	11.9
% of fish unaccounted for	8.4	11.6	17.6	16.8	3.6	12.8

Hematological results

Fish sampled from aquaria F_1#1, F_1#4, and F_1#5 were one month older than fish in the remaining aquaria. Hematocrit values for fish from aquaria F_1#1 (95.2 µg/liter Pb) were significantly lower at a 95% confidence interval than hematocrit values obtained from fish of aquaria F_1#4 and F_1#5. No statistical difference was found between fish of aquaria F_1#4 and F_1#5. Anemia, signified by low hematocrit and hemoglobin levels, would normally be expected where chronic lead poisoning has occurred

(Dawson; 1935, Farrelly and Pybus, 1969; and de Bruin, 1971). Fish sampled from aquaria F_1#2, F_1#3 and F_1C were one month younger than fish from the other three aquaria because of the difference in spawning and hatching dates. Analysis of variance and least significant difference tests showed no statistical difference existing in the hematocrit percentages for fish from F_1#2, F_1#3 and F_1C aquaria. The nonsignificance of the hematocrit values is probably indicative of the younger aged fish, which normally exhibit increase in hematocrit percentages with increased size and age, especially in the initial year of growth.

ACUTE BIOASSAY RESULTS

Hard Water Acute Bioassays

Flow-through bioassay

A hard water flow-through acute bioassay was conducted with a nominal high concentration of 10 mg/liter lead using rainbow trout with mean length and weight of 152 mm and 35.2 g respectively. The solution in the high concentration was milk white due to the complexation of lead as a carbonate. After 3 days one mortality occurred in the low concentration with a nominal lead concentration of 3.16 mg/liter. Upon examination, the fish was found to be diseased. After a total of 9 days no additional mortalities occurred and the test was terminated. A decision was made to perform an acute static test to determine the approximate toxicity of lead in hard water.

Static bioassay #1

A hard water static acute bioassay was conducted using six 35-liter aquaria. Ten rainbow trout with a mean length of 93 mm and weighing 8.2 g were placed in each concentration and a control aquarium. The nominal lead concentrations were, in mg/liter: 1000, 600, 500, 300, 100 and 50. One hundred percent mortality occurred within 1 hr at a concentration of 1000 mg/liter lead. The pH of this water was 4.55 and lies within a tolerable pH range for rainbow trout as stated by McKee and Wolf, 1963. Total mortality occurred in the 600 mg/liter lead aquarium at 18 hr. No mortalities occurred in the 500, 300, 100 and 50 mg/liter aquaria or the control after 8 days of exposure.

Table 13 gives water chemistry findings determined from each of the aquaria. It can be noted that the hardness of about 385 exists for all aquaria, whereas, the alkalinity and pH decrease as the lead concentration increases. This results from the precipitation of carbonates as lead carbonate. One can readily see why the alkalinity (a measurement of the carbonate system) is reduced with the precipitation of carbonates from solution. The decrease in pH results from the release of hydrogen ion which was tied up in the bicarbonate buffering system as HCO_3^-.

Table 13. Chemical results for static hard water acute bioassay #1.

Pb nominal concentration	1000	600	500	300	100	50	Control
Hardness (mg/liter)	386	384	382	388	386	384	384
Alkalinity (mg/liter)	2	5	30	118	218	240	267
pH	4.55	6.11	7.12	7.78	7.99	8.01	8.15
Temperature (^{o}C)	12.0	11.0	11.5	12.0	11.5	11.0	11.0
Dissolved oxygen (mg/liter)	9.2	8.6	8.6	9.4	9.4	9.2	9.0

It is rather obvious that acute lead toxicity occurs from the amount of free lead to which fish are exposed and not the complexed or precipitated lead species as demonstrated by no mortality occurring in 500 mg/liter lead and 100% mortality with 600 mg/liter. Atomic absorption analysis of the various lead concentrations showed a tremendous variation from one sampling period to another. A large portion of this variation undoubtedly resulted from a disproportionate sampling of free, colloidal and precipitated species of lead in the agitated aquaria. This variance was reduced by collecting water samples in beakers which would stand for 45 minutes to allow the precipitated forms to settle out of solution, at which time 10 ml of water was pipetted off the surface, acidified and analyzed by atomic absorption. Table 14 gives the results of three consecutive sampling periods collected in this manner. Note the daily decrease in the observed lead concentration with time. This could largely result from the aggregation of colloidal lead forms within the aquaria over time to form precipitates which settle out of solution. As stated previously, formation of lead precipitates is a slow process. This occurrence could be visualized in the aquaria themselves. Initially, upon the addition of lead nitrate, all the aquaria were very milky. The 500, 600 and 1000 mg/liter aquaria became quite clear within 3 hr with a large amount of lead carbonate precipitated on the aquaria bottoms. The aquaria with lead concentrations of 50, 100 and 300 mg/liter remained cloudy during the 8 days of the experiment but the cloudiness diminished daily.

Table 14. Lead analysis results (in mg/liter) of static hard water acute bioassay #1. 1/

Samples	Aquarium no. = Nominal Pb concentration					
	1000	600	500	300	100	50
1	220	17.0	1.0	15.7	23.3	3.87
2	---	---	0.50	8.52	6.17	2.60
3			0.27	2.05	1.15	0.70

1/ Analyzed by atomic absorption and therefore does not represent free lead values.

Static bioassay #2

On the basis of the first hard water static bioassay, it would appear that a flow-through acute bioassay would require a high concentration of 600 mg/liter lead. The stock solution concentration was computed to be 1256 g/liter at a 50% dilution ratio and 1666 g/liter at a 33% dilution ratio with our proportional diluter. These stock solution concentrations greatly exceed the solubility of lead nitrate in water (376.5 g/liter at 0°C). Running a flow-through acute bioassay in hard water would be totally impractical both from the standpoint of having to redesign our proportional diluter in order to achieve the desired high concentration but also because of the wide range of concentrations obtained with either a 50% or 33% dilution ratio.

Due to the above problem, it was decided that a narrow-range static bioassay would be much more practical and also serve to delimit the concentration range at which mortalities occur. It is recognized that the lead concentration mortality levels would be somewhat less with a flow-through bioassay than with a static bioassay but considering the wide concentration range of the flow-through bioassay, the latter is considered to provide the most useful information. The experiment consisted of five 35-liter aquaria with lead concentrations of 500, 520, 540, 560 and 580 mg/liter and a control aquarium. Ten rainbow trout with a mean length and weight of 85.9 mm and 5.9 g respectively, were acclimated to hard water for 8-days and placed in each of the experimental aquaria.

Water chemistry data was similar to that of the previous test. Hardness (385 mg/liter) was essentially the same for all aquaria. The M.O. alkalinity decreased from 25 mg/liter (500 mg/liter Pb) to 6 mg/liter (580 mg/liter Pb). The pH decreased from 7.17 to 6.23 with increased lead

concentration. The temperature ranged from 13.8°C to 14.2°C, and dissolved oxygen ranged from 8.4 to 9.0 mg/liter. The acute bioassay ran for 96-hr at which time it was terminated because of no additional mortalities occurring during the last 24-hr (Eaton, 1970). The 96-hr mortality distribution was as follows: 500 mg/liter lead (0%), 520 mg/liter lead (0%), 540 mg/liter lead (30%), 560 mg/liter lead (100%) and 580 mg/liter lead (100%) (Table 15). A TL_{50} (96-hr) value of 542 mg/liter was determined by a log-probit method (Sprague, 1969).

Table 15. Lead analysis(in mg/liter) and 96-hr mortality results for static hard water acute bioassay #2.

Samples (Days post sampling)	Aquarium no. = Nominal Pb concentration				
	500	520	540	560	580
1	1.28	0.85	1.13	6.42	8.75
2	1.53	0.56	1.05	6.32	8.42
3	0.54	0.36	0.50	4.26	4.64
4	0.52	0.17	0.47	4.17	4.45
$\overline{X}$	0.97	0.48	0.79	5.29	6.54
% Mortality	0	0	30	100	100

Table 15 gives the analytical results for lead as determined by atomic absorption spectrophotometry. As seen previously, the concentration decreases daily, probably as a result of the slow precipitation of lead. These results are assumed to approach free lead values for the aquaria containing nominal lead concentrations of 520, 540, 560, and 580 mg/liter. As was stated previously, atomic absorption results for lead in soft water give essentially free lead results because of the high solubility of lead in this water. In a general sense, the analysis for lead in hard water in which the carbonate buffering capacity has been eliminated by the precipitation of lead carbonate, is analogous to atomic absorption lead analysis in soft water. However, such analyses cannot be construed as actually free lead, since pulse polarographic analysis was not performed to substatiate this. The mean concentrations for the four sampling periods is given in Table 15. Using these data, a log-probit plot (Sprague, 1969), gave a TL_{50} concentration of 1.00 ppm lead in 96-hrs. A nominal TL_{50} lead concentration of 542 ppm was determined by the same procedure.

(See Appendix E for discussion on the nature of lead in hard water as analyzed by atomic absorption spectrophotometry).

Static bioassay #3

A third hard water static bioassay was performed for the purpose of obtaining and comparing pulse polarographic free lead results with analytical results obtained by atomic absorption spectrophotometry. The bioassay was run in 35-liter aquaria placed in a Min-o-cool tank which served as a water jacket for maintaining a temperature of 7°C. The soft water acute bioassay (yet to be reported) was conducted at a temperature of 7°C. Therefore, both experiments were run at comparable temperatures. Ten rainbow trout with a mean length and weight of 132 mm and 24 g respectively, were placed into each of six static aquaria eight hours after the introduction of the toxicant. This allowed for an initial equilibrium-free lead level to be achieved and minimized toxicant shock to the fish. The nominal lead concentrations tested were 500, 490, 480, 470, 460 mg/liter lead and a control aquarium.

Water chemistry data is presented in Table 16. It will be noted that the hardness (290 mg/liter) and alkalinity (228 mg/liter) in the control aquarium is lower than that in the static hard water bioassays 1 and 2, in which the hardness was 384 mg/liter and the alkalinity was 267 mg/liter. These differences lie within the natural seasonal range found in the hard water source as shown in Table 2. As was found in the previous two static bioassays, the alkalinity and pH decrease with increased lead concentrations and the hardness remains relatively unchanged. As would be expected, it can also be seen that conductivity increases with increased lead concentrations (Table 16).

Table 16. Chemical results for static hard water acute bioassay #3

Pb nominal concentration	500	490	480	470	460	Control
Hardness (mg/liter)	300	300	300	300	300	290
Alkalinity (mg/liter)						
phth	0	0	0	0	0	9
M.O.	7	9	10	20	21	228
pH	6.89	6.91	6.97	7.08	7.26	8.78
Conductivity	1480	1460	1440	1420	1400	1080
Temperature (°C)	7.0	7.0	7.0	7.0	7.0	7.0
Dissolved oxygen (mg/liter)	9.8	9.4	9.8	9.4	9.2	9.8

Water samples for lead analysis were collected daily in 4 oz Nalgene bottles which were sealed, placed in a plastic bag filled with experimental water, and maintained at the 7°C bioassay temperature. Upon termination of the experiment, the samples for the four day sampling period were allowed to stand undisturbed to permit settling of lead carbonate precipitate at which time aliquot samples were pipetted off the surface and pooled for pulse polarographic and atomic absorption analysis. The pooled samples were analyzed by four procedures: 1) pulse polarography, 2) atomic absorption spectrophotometry: a) unfiltered, b) filtered 0.45μ (Millipore filter), and c) filtered 0.025μ (Millipore filter). The analytical results for lead are given in Table 17.

Table 17. Lead analysis (in mg/liter) and 96-hr mortality results for static hard water acute bioassay #3.

	Aquarium no. = Nominal Pb concentration				
	460	470	480	490	500
Analytical procedure					
1. Pulse polarography	0.29	1.26	2.77	4.79	7.18
2. Atomic absorption					
a. unfiltered	0.25	1.30	2.85	5.05	7.56
b. 0.45μ filtered	0.103*	0.545*	--	0.380*	--
c. 0.025μ filtered	0.005*	0.805*	--	0.465*	--
% Mortality	0	30	100	100	100

* Atomic absorption analysis utilizing a modified APDC - MIBK extraction technique (Fishman and Midgett, 1968).

It can be noted from the analytical data (Table 17) that in the analysis of lead in hard water, where the carbonate buffering capacity of the water has been eliminated by the addition of lead, pulse polarography and atomic absorption results are quite comparable. It is also evident that the filtration of water sample through either 0.45μ or 0.025μ filters removes free lead from the water and gives inconsistent and erratic results. Marvin, Proctor and Neal, 1970, reported similar finding in analyzing for copper in filtered fresh and seawater samples.

The bioassay ran for a period of 96 hours at which time the test was terminated because of no additional mortalities occurring during the last 24 hours of the experiment (Eaton, 1970). The mortality distribution is

given in Table 17. The nominal lead concentration giving 50% mortality occurred between 470 ppm lead with 30% mortality and 480 ppm lead with 100% mortality. A log-probit method of analysis (Sprague, 1969), gave a nominal 96-hr TL_{50} of 471 mg/liter lead and a free lead 96-hr TL_{50} of 1.38 mg/liter.

Soft Water Acute Bioassay

A soft water flow-through acute bioassay was conducted using a 50% dilution ratio with a high nominal concentration of .50 mg/liter lead. Rainbow trout with a mean length and weight of 161 mm and 43.0 g respectively, were acclimated to soft water with a mean hardness of 30 mg/liter for 2 weeks. Ten fish were acclimated in duplicate 35-liter aquaria for each concentration of lead for 3 days prior to the addition of toxicant.

Water chemistry results gave the following mean values: hardness (30.0 mg/liter), alkalinity (29.3 mg/liter), pH (6.85), conductivity (165 µhos/cm), and temperature (7.0°C). The mean dissolved oxygen was 8.36 mg/liter and ranged from 6.4 to 9.8. Table 18 gives the mean lead concentrations analyzed by atomic absorption and percent mortality for each duplicate concentration. The TL_{50} value was calculated utilizing a computerized log-probit method of analysis from the lethal threshold concentration and was found to be .14 mg/liter over an 18-day period(Daum, 1969; Daum and Killcreas, 1966). The fish were fed an appropriate ration during the experiment because of the extended time period over a routine 96-hr acute.

Table 18. Lead analysis in mg/liter and mortality results for soft water acute bioassay.

	Aquaria										
	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	Ca
Mean lead concentration	.55	.52	.26	.28	.16	.13	.077	.067	.1	.03	0
% mortality	100	100	70	90	40	27	0	20	0	0	0

APPLICATION FACTORS FOR HARD AND SOFT WATER BIOASSAYS

Hard Water Application Factor

As explained previously, the lead analyses in hard water by atomic absorption spectrophotometry are relatively meaningless because of the inability

of knowing what the nature (free, colloidal or precipitated) of lead measured in a sample actually is. This situation leaves two avenues by which the application factor can be approached. These are: (1) we know the total amount of lead added (i.e., the nominal concentration), and (2) the pulse polarography analysis of free lead in the chronic hard water growth bioassay. Table 19 summarizes this analytical data for lead that is of particular concern for calculating the hard water application factor.

Table 19. Summarized analytical data for lead in the hard water chronic bioassay.

Lead concentration (mg/liter)	Aquaria					
	H-1	H-2	H-3	H-4	H-5	H-C
Nominal concentration	3.24	1.08	0.36	0.12	0.04	0.00
Free lead concentration (pulse polarography)	.064	.044	.032	.018	.011	.003

The application factor is determined by dividing the maximum acceptable toxicant concentration ("MATC") of the hard water chronic bioassay by the TL_{50} concentration of the acute bioassay (Mount and Stephan, 1967b).

Nominal lead (NL) concentration application factor

The "MATC" of the hard water chronic bioassay was found to occur between aquarium H-4, with a no effect nominal concentration level of 0.12 mg/liter, and aquarium H-3 (0.36 mg/liter Pb) in which fish exhibited the "blacktail" effect. The 96-hr TL_{50} nominal concentration at the lethal threshold in the static acute bioassay #3 was 471 mg/liter lead. The hard water nominal lead application factor is calculated to lie between .0002 and .0008.

$$\text{Hard Water Application Factor (NL)} = \frac{\text{"MATC"}}{TL_{50}\ (96\text{-hr})} =$$

$$\frac{0.12\ \text{mg/liter}}{471\ \text{mg/liter}} \text{ and } \frac{0.36\ \text{mg/liter}}{471\ \text{mg/liter}}, \text{ or from .0002 to .0008}$$

Free lead (FL) concentration application factor

The "MATC" on a free lead basis lies between aquarium H-4 (.018 mg/liter) and H-3 (.032 mg/liter). The free lead 96-hr TL_{50} concentration was

1.38 mg/liter. Therefore, the hard water, free lead application factor is calculated to lie between .0130 and .0232.

$$\text{Hard Water Application Factor (FL)} = \frac{\text{"MATC"}}{TL_{50}\ (96\text{-hr})} =$$

$$\frac{0.018\ \text{mg/liter}}{} \quad \text{and} \quad \frac{0.032\ \text{mg/liter}}{1.38\ \text{mg/liter}}, \text{ or from } .0130 \text{ to } .0232$$

Soft Water Application Factor

Table 20 summarizes the analytical data for lead in the soft water F_1 generation reproduction bioassay. Since we consider the nominal and free lead concentration to be the same for all practical purposes, in soft water, the "MATC" occurred between aquaria S-5 (6 µg/liter), no effect level, and S-4 (11.9 µg/liter) in which four fish exhibited the "black-tail" effect. An 18-day soft water flow-through acute bioassay gave a lethal threshold TL_{50} value of 140 µg/liter.

Table 20. Summarized analytical data for lead in the soft water F_1 generation reproduction bioassay.

Lead concentration (µg/liter)	Aquaria 1	2	3	4	5	C
Nominal concentration	100.0	50.0	25.0	12.5	6.2	0.0
Free lead concentration (atomic absorption)	95.2	47.6*	23.8*	11.9*	6.0*	3.1**

* Computed from analyzed high concentration.
** Determined by pulse polarography.

Free lead (FL) concentration application factor

The soft water free lead application factor is calculated to lie between .0429 and .0850.

$$\text{Soft Water Application Factor (FL)} = \frac{\text{"MATC"}}{TL_{50}\ (18\ \text{day})} =$$

$$\frac{6\ \mu\text{g/liter}}{140\ \mu\text{g/liter}} \quad \text{and} \quad \frac{11.9\ \mu\text{g/liter}}{140\ \mu\text{g/liter}}, \text{ or from } .0429 \text{ to } .0850.$$

TESTING VALIDITY OF APPLICATION FACTOR APPROACH AS APPLIED TO FISH IN DIFFERENT WATER QUALITIES

Table 21 summarizes elements of the application factor data needed to test the applicability of the application factor concept to toxicity in different water qualities.

Table 21. Summary of application factor data.

	Hard water		
	Nominal lead Concentration	Free lead concentration	Soft Water
"MATC"	0.12 to 0.36 mg/liter	.018 to .032 mg/liter	6.0 to 11.9 μg/liter
TL_{50} (lethal threshold)	471 mg/liter	1.38 mg/liter	140 μg/liter
Application factor	.0002-.0008	.0130-.0232	.0429-.0850

Figure 11 gives the schematic for testing the application factor concept which would permit chronic data developed for one water quality to be used to approximate the maximum acceptable toxicant concentration of another quality of water for which only acute toxicity data are available.

Test of Application Factor Approach as Applied to Nominal Lead Concentration in Hard and Soft Water

(1) Soft Water Application Factor x (NL) TL_{50} Hard Water Acute ≈ (NL) "MATC" For Hard Water (0.12-0.36 mg/liter)

.0429 - .0850 x 471 mg/liter = 20.21 - 40.04 mg/liter

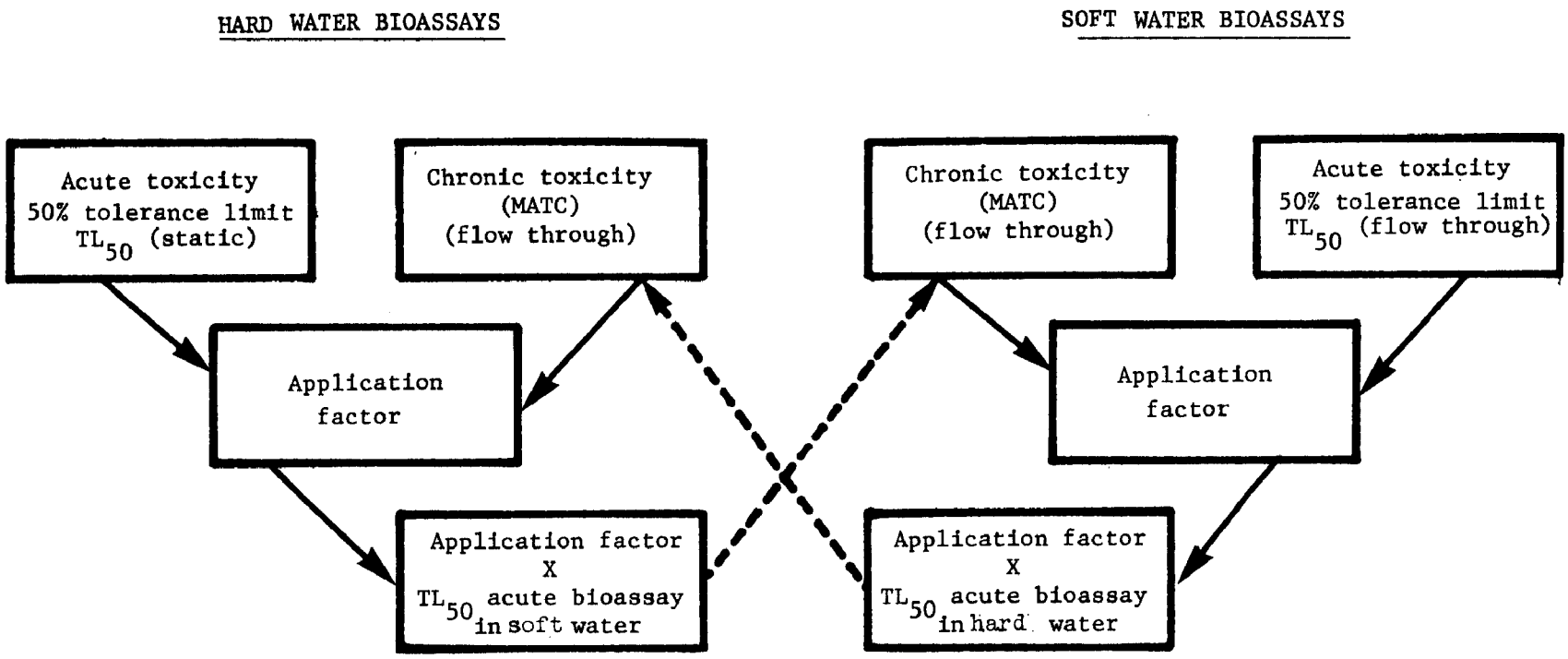
Test: INVALID (Computed "MATC" range, off by a factor of 56 to 334)

(2) Hard Water Application Factor (NL) x TL_{50} Soft Water Acute ≈ "MATC" for Soft Water (6.0-11.9 μg/liter)

.0002 - .0008 x 140 μg/liter = .03 - .11 μg/liter

Test: INVALID (Computed "MATC" range, off by a factor of 54 to 397)

Figure 11. Lead toxicity to rainbow trout and validity of application factor.



Test of Application Factor Approach as Applied to Free Lead Concentration in Hard and Soft Water

(1)	Soft Water Application Factor	x	(FL) TL_{50} Hard Water Acute	$\approx$	(FL) "MATC" for Hard Water (.018-.032 mg/liter)
	.0429 - .0850	x	1.38 mg/liter	=	.059 - .117 mg/liter

Test: VALID -"MATC" approximated (Computed "MATC" range off by a factor of 1.8 to 6.5)

(2)	Hard Water Application Factor (FL)	x	TL_{50} Soft Water Acute	$\approx$	"MATC" for Soft Water (6.0-11.9 µg/liter)
	.0130 - .0232	x	140 µg/liter	=	1.8 - 3.3 µg/liter

Test: VALID - "MATC" approximated (Computed "MATC" range off by a factor of 1.8 to 6.6)

Discussion of the Application Factor Approach as Applied to Different Water Qualities

The application factor concept appears to be functional with heavy metals, such as lead, which exhibit a complexing behavior in different water qualities only if analysis for the free or soluble metal is employed. This analytical limitation poses a serious and unresolved problem if the application factor concept is to find widespread use. The determination of "MATC" and acute TL_{50} concentrations would require analysis of the dissolved metal. In addition, water samples collected to monitor pollutant concentrations of a particular water, would necessitate the determination of free metal ions if such results were to be compared to previously determined "MATC" values.

Most agencies monitoring heavy metal stream pollution problems use atomic absorption spectrophotometry as the primary instrument for metal analysis. Sample filtration does not appear to be a reliable method for determining dissolved metals by atomic absorption spectrophotometry. Filtration as a method for approximating free metal analysis will require experimental verification with many complexing metals before the procedure could be considered reliable. Until that time, atomic absorption spectrophotometry should not be expected to provide data useful in deriving or implementing application factor data where the complexing of heavy metals occurs.

Section VI

ACKNOWLEDGMENTS

Dr. W. Harry Everhart, Chairman Fishery Major of the Department of Fishery and Wildlife Biology at Colorado State University, was Project Director. Colorado Division of Game, Fish and Parks provided personnel (John Goettl, James Sinley, and Norwin Smith), and the bioassay facilities at the Fort Collins Research Center. Dr. Janet Osteryoung, Quantitative Chemist with Colorado State University, performed the pulse polarographic analysis of the lead water samples and constructed the solubility diagrams for lead in soft and hard water.

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Section VII

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Toxicity of lead to rainbow trout. (In preparation).

Davies, Patrick H. Testing the application factor approach as applied to different water qualities. (In preparation).

Section IX

APPENDICES

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Appendix A. Discussion of the complexities associated with analyzing the character of lead in water.

Appendix B. Discussion of the problems associated with the analysis of lead by atomic absorption spectrophotometry.

Appendix C. Discussion of the pH problems associated with the analysis of lead by pulse polarography.

Appendix D. Atomic absorption analysis of feed.

Appendix E. Discussion on the nature of lead in hard water as analyzed by atomic absorption spectrophotometry.

APPENDIX A

DISCUSSION OF THE COMPLEXITIES ASSOCIATED WITH ANALYZING THE CHARACTER OF LEAD IN WATER
(personal communications, Dr. Janet Osteryoung)

Equilibrium calculations show that the total solubility of lead in hard water is about 30 μg/liter and 500 μg/liter in soft water (Figures 12 and 13). The solubilities and distribution diagrams for the different lead species in hard and soft water are intended to be suggestive rather than definitive. In each figure the arrow indicates the pH of the water in the test aquaria (used data from H-1, hard water high concentration, and S-1, soft water high concentration). The curve, C_{Pb}, gives the solubility of lead in solution. Soluble complexes such as $PbNO_3^+$, not shown, were omitted because they were found to be unimportant in that they represent an insignificant fraction of the total species present. All values are computed at 25°C because of the general lack of equilibrium constant data at the different bioassay temperatures which exhibited large seasonal fluctuations, ranging from 5°C to 21°C. By reason of the resulting inaccuracies existing inherently by assuming a temperature of 25°C, no activity corrections were made. All equilibrium constants are thermodynamic values. In any case, should such correction have been made, they would be small due to the ionic strengths in soft and hard water which are only 1-2 and 10-20 m<u>M</u>, respectively.

In soft water with a pH $\leq$ 5.4, $PbSO_4$ is present and limits the lead concentration in solution (Figure 13). Above pH 5.4, a situation characterized in the test aquaria, $PbCO_3(s)$ and $Pb_2(OH)_2CO_3(s)$ are present and limit the lead concentration. In hard water a pH > 6.0 is the level at which $PbCO_3(s)$ and $Pb_2(OH)_2CO_3(s)$ are present and cause a corresponding limitation (Figure 12). Under experimental conditions, the most important factor determining lead solubility in both these waters is the carbonate concentration which in turn depends on the partial pressure of $CO_2(g)$ and the pH. Both pH and $CO_2(g)$ concentrations are subject to large local fluctuations due to fish respiration, resulting in an extremely sluggish $CO_2(g) \rightleftarrows CO_2(aq)$ equilibrium. In addition, the equilibria involving Pb (II) precipitation and dissolution are very slow. Consequently, the equilibrium calculations only indicate permissible ranges and are not necessarily an accurate description of the system, even if they could be done completely and accurately.

The large standard deviations that exist for both analytical methods used in determining lead concentration (pulse polarography and atomic absorption) are believed not to be due to the methods of analysis, and probably not due to the diluter system other than on those occasions when a diluter malfunction occurred. They probably arise through local changes in solution conditions in the aquaria which cause precipitation of lead or solution of suspended material. One factor which would greatly influence this is temperature. Since temperature was not held constant and fluctuated

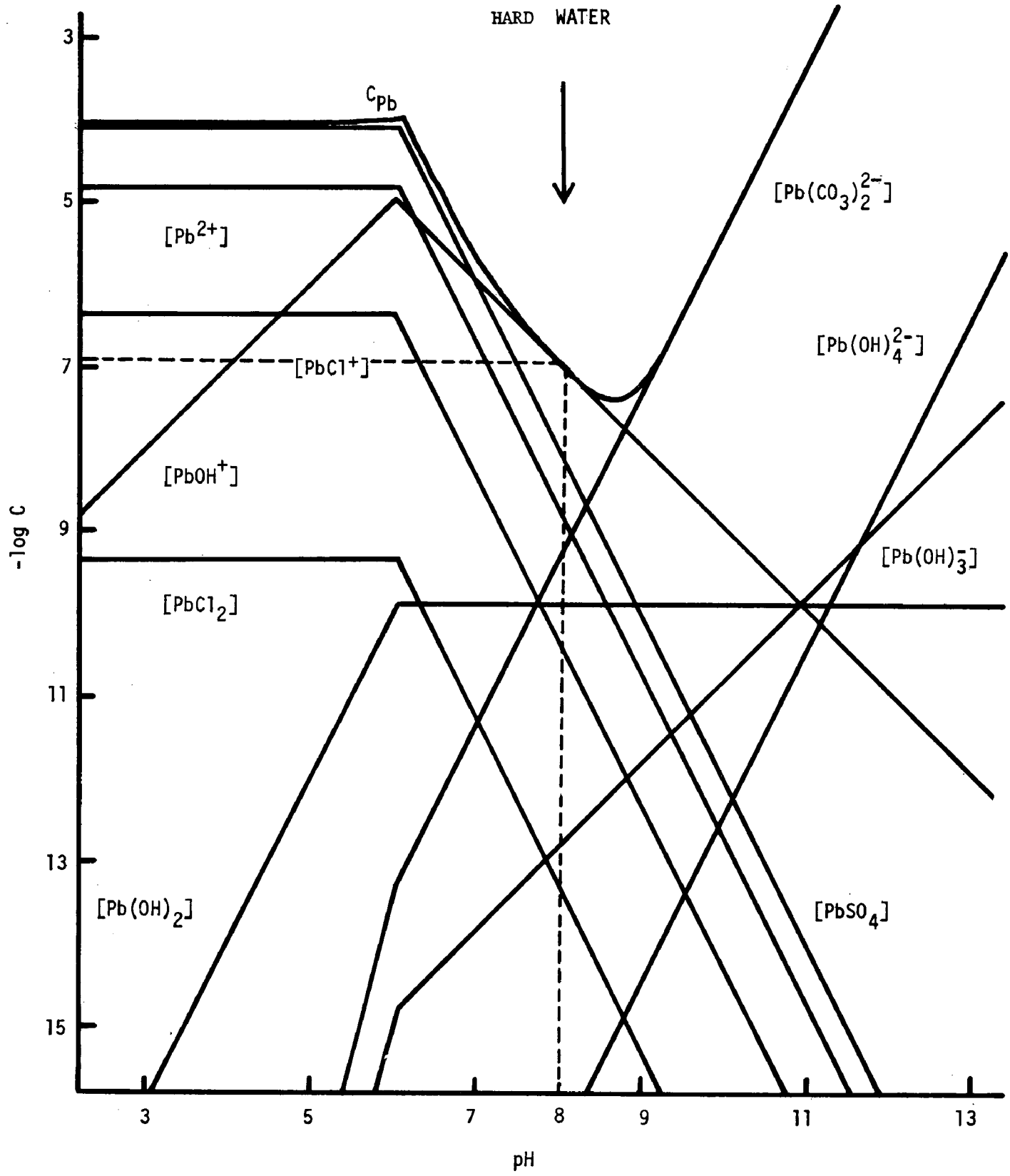


Figure 12. Solubility and species distribution for Pb (II) in hard water. Arrow indicates average pH in aquarium H-2 (Stumm and Morgan, 1970).

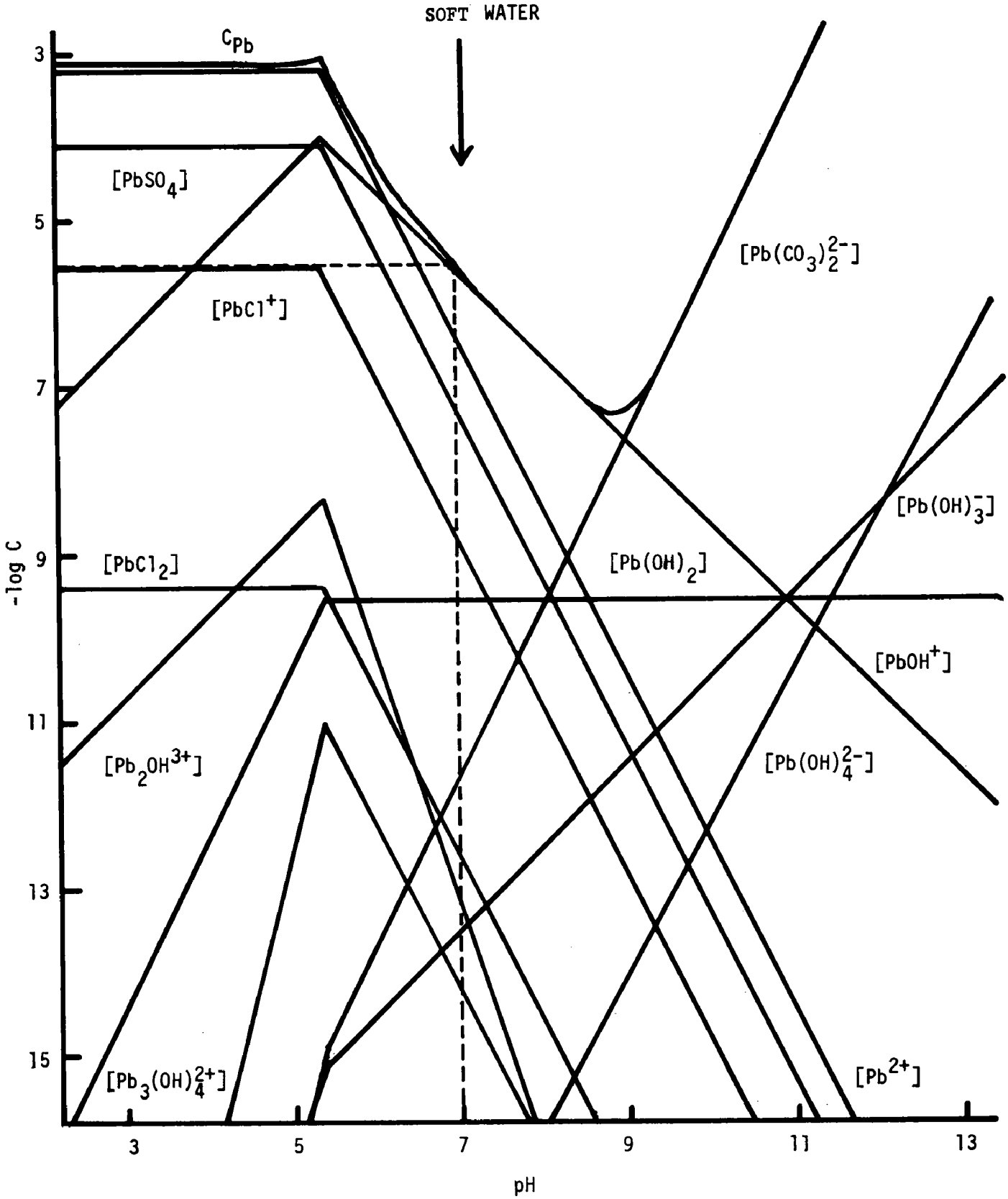


Figure 13. Solubility and species distribution for Pb (II) in soft water. Arrow indicates average pH in aquarium S-2 (Stumm and Morgan, 1970).

seasonally with a range of $5^{o}C$ to $21^{o}C$, the rate of colloid and precipitate formation would most certainly be affected. This situation would exert the greater influence on atomic absorption results where analysis was performed soon after sampling. Another factor which might contribute to the variance of lead results is the effect of metabolic byproducts, particulate matter, and algal growths on the various lead species in the test solutions.

APPENDIX B

DISCUSSION OF THE PROBLEMS ASSOCIATED WITH THE ANALYSIS OF LEAD BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

Relatively speaking, atomic absorption analysis of lead in soft water approximates total lead present, and in this case also the amount of free lead because of the high solubility (500 μg/liter) of lead in soft water. It is somewhat erroneous to speak of lead analysis being a total analysis when dealing with a highly alkaline or hard water, such as the well water used in this study. This assumes that such an analysis would measure free, colloidal and precipitated forms of lead in an amount proportional to their actual existence in an aquarium. Such an assumption is particularly invalid with regard to precipitated lead forms which would tend generally to accumulate on the bottom of an aquarium and not remain uniformly distributed in the water. In addition, with the proportional dilutor system continuously supplying new solutions of lead, there would be a continuous buildup in the aquarium of precipitated lead forms which would greatly exceed that encountered in a static situation.

The above factors are further complicated by respiratory and metabolic by-products, and the effect of particulate matter and algal growth in the aquarium on the character of lead in such solutions. Analytical results by atomic absorption in hard water are relatively meaningless because of the inability to define the nature and character of the lead species sampled. For these reasons, pulse polarographic analysis which measures free lead, and the nominal lead concentrations, the known added amount of lead, provides the only definable knowledge we have of the hard-water lead system.

APPENDIX C

DISCUSSION OF THE PH PROBLEMS ASSOCIATED WITH THE ANALYSIS OF LEAD BY PULSE POLAROGRAPHY

(personal communications, Dr. Janet Osteryoung)

As previously stated, about 60% of the samples analyzed by direct analysis or by the addition of LiCl gave no signal for lead. A lead signal was obtained upon acidification of these samples. Several tests of standard lead solutions in the range 2×10^{-7} to 1×10^{-5} M (0.04 - 2 mg/liter) containing 1×10^{-3} to 4×10^{-2} M KNO_3, $NaNO_3$, LiCl, or KCl, showed that the pulse polarography signal (i_p) in "neutral" solutions is at least a factor of two less than in acid solutions (pH $\leq$ 4). The decrease in signal is apparently most pronounced at lower lead levels. For example, "neutral" solutions containing 0.105 mg/liter lead give no signal, but acid solutions give a clearly defined signal, which was a factor of six above the limiting instrument sensitivity. Experiments employing $HClO_4$, HCl, and HNO_3 as the acid, convincingly demonstrate that pH rather than the choice of anion controls this effect. However, it is not possible to investigate this phenomenon in buffered solutions because of the effect of the ionic strength and the complexing behavior of the anions present on the signal. Here we are talking about the anions associated with a buffered system and not those associated with the particular acid used. Of the acids used, the ClO_4^- and NO_3^- anions would exhibit no complexing behavior, and the effect of Cl^- would be only slight compared to that of a buffered system. Therefore, it appears almost impossible to do a reliable study on the effect of pH.

The question of the effect of acidity on the magnitude of the signal is serious and unresolved. Experiments on standard solutions show conclusively that the problem does not occur because of changes in the rate of lead reduction with changes in medium, for the same phenomenon is observed both with differential and normal pulse polarography. Lower signals in an unbuffered neutral solution are due in part to simultaneous reduction of residual oxygen which consumes hydrogen ion and produces a local region of high pH near the indicator electrode. This in turn, can cause local precipitation of $Pb(OH)_2$. However, this effect should be much more pronounced in the differential than in the normal pulse mode. Since the observed signal decrease is nearly the same in the two modes, local pH changes cannot be entirely responsible.

In normal pulse polarography, the magnitude of the diffusion current depends only on the concentration and the diffusion coefficient of the substance being reduced. Equilibrium calculations show, for the standard solutions investigated, that there should be no problem with insolubility in the bulk solutions, and therefore the concentration should not vary as a function of pH. This leaves the diffusion coefficient as the only

factor capable of influencing the magnitude of the diffusion current. When changing pH from 7 to 4, the predominant lead species change from $PbOH^+$ to Pb^{++} respectively; it is surprising that the respective diffusion coefficients should vary by a factor of four (i_d is proportional to D 1/2) or that the factor should increase with dilution. This, however, seems to be the most reasonable hypothesis in the absence of further data.

A question that legitimately could be asked is: Why, with acidification of samples, are pulse polarography results reported as soluble and not total lead? The samples were acidified immediately prior to the running of a polarogram. It would appear that insufficient time existed for the conversion of the undissociated (colloidal and precipitated forms) to dissociated or soluble species of lead. Yet, with the resulting lower pH, the diffusion coefficients of the soluble species present were increased by changing the predominant lead species from $PbOH^+$ to Pb^{++}, thus giving an adequate lead signal.

APPENDIX D

ATOMIC ABSORPTION ANALYSIS OF FEED

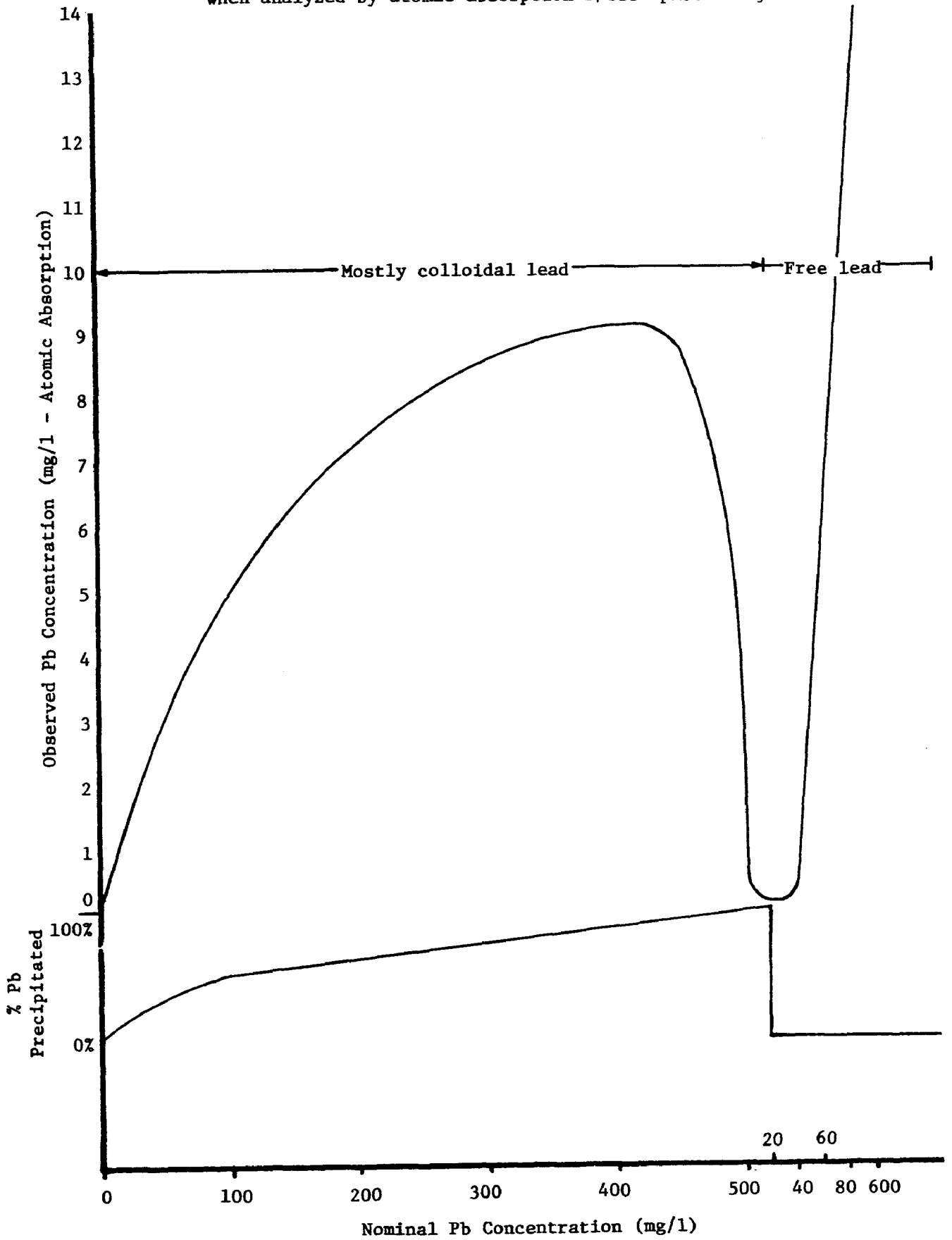
Feed size	Zn (μg/g)	Cu (μg/g)	Pb (μg/g)
#0 crumbles	75.60	11.15	<2.5
#1 crumbles	91.59	15.25	<2.5
#2 crumbles	106.34	18.16	<2.5
#3 crumbles	131.81	17.96	<2.5
#4 crumbles	114.35	16.67	<2.5
1/8" pellets	96.85	23.57	<2.5
7/32" pellets	100.48	23.45	<2.5

APPENDIX E

DISCUSSION ON THE NATURE OF LEAD IN HARD WATER AS ANALYZED BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

As seen from Figure 14, the observed lead concentration when analyzed by atomic absorption spectrophotometry is mostly of a colloidal nature (in a hard water with an alkalinity of 267 mg/liter) for nominal lead additions from 0 to 500 mg/liter. The graph as drawn would represent some point in time, let us say 6 to 24 hr, after lead is added to the hard water. Lead would exist mostly free in solution above that point in which the added lead eliminates the carbonate system, at about 520 mg/liter of added lead. This is also the point corresponding to 100% precipitation of lead; above this point lead additions would go freely into solution. The graph is intended to be descriptive and not represent what definitely occurs in the lead hard-water system. The graph does not, nor is it intended to depict possible changes that might occur in the whole anion and cation system resulting from decrease in pH due to the addition of lead which releases hydrogen ions from the bicarbonate system, or from absorption of atmospheric carbon dioxide by the water, which would tend to replenish carbonates.

Figure 14. Graphic interpretation of the nature of lead in well water when analyzed by atomic absorption spectrophotometry.



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EFFECTS OF CHEMICAL VARIATIONS IN AQUATIC ENVIRONMENTS: Lead toxicity to rainbow trout and testing application factor concept.

10 Author(s)

Davies, Patrick H., and W. Harry Everhart

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*Rainbow trout, *lead, *toxicity

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*Rainbow trout, *lead, *toxicity
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27 Abstract

Four chronic bioassays were conducted to determine the toxicity of lead to rainbow trout. Results obtained from acute and chronic bioassays in hard water (alkalinity 243.1 mg/liter) and soft water (alkalinity 26.4 mg/liter) were used to test the application factor approach as related to different water qualities. The toxicity of lead to rainbow trout in hard water was determined on a total and dissolved lead basis. The 96-hr TL_{50} and "MATC" on a total lead basis were 471 mg/liter and 0.12 to 0.36 mg/liter respectively, which yielded an application factor of .0002 to .0008. Analysis of the free or dissolved lead gave a 96-hr TL_{50} of 1.38 mg/liter and a "MATC" of 0.018 to 0.032 mg/liter, resulting in an application factor of .0130 to .0232. Total and free lead were considered to be the same in soft-water. The 18-day TL_{50} and "MATC" obtained from the soft water bioassays were 140 µg/liter and 6.0 to 11.9 µg/liter lead respectively. Computations using the TL_{50} and "MATC" values gave a soft water application factor of .0429 to .0850. The maximum acceptable toxicant concentration ("MATC") was determined in both hard and soft water bioassays on the occurrence of abnormal black tails caused by chronic lead exposure. The application factor approach as related to different water qualities was found to be very promising when lead analysis was limited to the free or dissolved metal and failed when total hard water lead concentrations were used.

Abstractor W. Harry Everhart | Institution Cornell University