

AN ABSTRACT OF THE THESIS OF

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Title Estimation of Chronically Safe Zinc Concentrations with

Steelhead Trout (*Salmo gairdneri*) Emphasizing Ventilatory Responses

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Richard A. Tubb

A 96 hr toxicity test, measuring ventilation rate, was developed to estimate safe zinc concentrations for steelhead trout (*Salmo gairdneri*). Ten short-term exposures were conducted with zinc and steelhead trout at different regimes of water temperature (7, 12, and 17 C), total hardness (25 and 125 mg/l as CaCO₃), and steelhead strain (Alsea and Santiam Rivers of Oregon). Ventilatory frequencies were enumerated from bioelectric potentials generated by buccal and opercular openings and closings as detected by non-implanted electrodes and recorded on a strip-chart recorder. Ventilatory rates showed significant increases at the highest test concentration in five of ten tests. Hematocrit and leucocrit values were inconsistent and highly variable between replicate tests. Respiratory responses indicated a 'safe' toxicant level of between 117 and 144 µg/l Zn when the water was at 12 C and a hardness of 25 mg/l. Ventilatory rate

measurements permitted detection of zinc below chronically 'safe' levels determined in embryo-early juvenile exposures (444-819 $\mu\text{g}/\text{l}$). This indicates that ventilation rates can probably be used in pre-market screening of new chemicals, developing water quality criteria, and biomonitoring of effluents.

Estimation of Chronically Safe Zinc
Concentrations with Steelhead Trout (Salmo
gairdneri) Emphasizing Ventilatory Responses

by

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TABLE OF CONTENTS

| | <u>Page</u> |
|--|-------------|
| INTRODUCTION | 1 |
| Need for Rapid Bioassay Methods | 1 |
| Research Objectives | 2 |
| LITERATURE REVIEW | 3 |
| Bioassay Methods Currently Used | 3 |
| Blood Parameters as Indicators of Sublethal Toxicity | 5 |
| Respiratory Monitoring to Detect Sublethal Toxicity | 6 |
| Zinc as an Aquatic Toxicant | 10 |
| METHODS AND MATERIALS | 12 |
| Test Design | 12 |
| Test Fish | 12 |
| Dilution Water | 14 |
| Toxicant | 17 |
| Embryo-Early Juvenile Exposure | 19 |
| Short-term Tests for Respiratory and Hematological Response | 23 |
| Respiratory Monitoring | 26 |
| Blood Parameters | 33 |
| Statistical Analyses | 34 |
| RESULTS | 36 |
| Embryo-Early Juvenile Test | 36 |
| Respiratory Monitoring | 36 |
| Blood Parameters | 41 |
| Response Levels | 41 |

| | <u>Page</u> |
|------------------|-------------|
| DISCUSSION | 43 |
| SUMMARY | 55 |
| LITERATURE CITED | 56 |

LIST OF FIGURES

| <u>Figure</u> | | <u>Page</u> |
|---------------|---|-------------|
| 1 | Diagram of Incubator Used in the Embryo-Early Juvenile Test | 21 |
| 2 | Diagram of Electrode Chamber Used in the Short-Term Zinc Exposures | 24 |
| 3 | Hardness Generator | 27 |
| 4 | Schematic Diagram of the Short-Term Testing System | 29 |
| 5 | Electrode Chambers with Cables Leading to the Recording Apparatus | 31 |
| 6 | Multiplex Switching Device | 31 |
| 7 | Examples of Ventilation Patterns from Four Test Fish Showing Varied Respiratory Rates. Peak Height is Determined by Ventilatory Amplitude, Recorder Sensitivity Setting, and Position of Fish in Relation to Electrodes | 38 |
| 8 | Examples of Ventilatory Patterns from Four Test Fish Showing Disruption of Regular Patterns in the Fish Exposed to the Low Zinc Concentration (D) | 46 |
| 9 | Plot of Zinc-Induced Changes in Mean Ventilatory Frequency, Expressed as Log_{10} | 48 |

LIST OF TABLES

| <u>Table</u> | | <u>Page</u> |
|--------------|---|-------------|
| 1 | Nominal Test Conditions for Short-Term Tests and Dates Completed | 13 |
| 2 | Fish Size in Short-Term Toxicity Tests | 15 |
| 3 | Measured Water Quality Parameters | 16 |
| 4 | Measured Zinc Concentrations (ug/l) in Embryo-Larval and Short-Term Exposures | 18 |
| 5 | Percent Mortalities from Embryo-Early Juvenile Test | 37 |
| 6 | Results of Ventilatory Rate Changes, Estimated Detection Ranges, and Mean Control Period Rates | 40 |
| 7 | Results of Blood Parameter Changes and Detection Ranges | 42 |

ESTIMATION OF CHRONICALLY SAFE ZINC CONCENTRATIONS WITH
STEELHEAD TROUT (Salmo gairdneri) EMPHASIZING VENTILATORY RESPONSES

I. INTRODUCTION

A. Need for Rapid Bioassay Methods

Evaluations of hazards resulting from the production and use of chemicals are difficult judgements that must be based on the best scientific evidence available. The accelerated rate of new chemical production, coupled with recent legal mandates for control, intensify the need for toxicity tests to set permissible discharge limits and test new substances prior to marketing. Growth of the chemical industry in the twentieth century has increased the number of known chemicals to more than two million. Approximately 250,000 new chemicals are produced annually and about 1,000 reoccur in the natural environment (Maki 1979). The surface waters of the earth, an integral part of the environment, obviously have a finite capacity for assimilation of waste substances. Serious water quality degradation is the ultimate consequence if chemical production and distribution are not controlled.

During the 1970's, many laws were enacted to deal with environmental pollution. The Federal Water Pollution Control Act Amendments of 1972 (P.L. 92-500) mandates the Environmental Protection Agency to set water quality standards for toxicants in surface waters. P.L. 92-500 also directs the EPA administrator to require biological monitoring of any appropriate point source to fulfill the objectives of this Act. The Toxic Substances Control Act of 1976 (P.L. 94-469) requires evaluation of all aspects of production, pre-market testing, distribution, and disposal of chemicals (Train 1978). These and other laws (e.g. Clean Water Act Amendments of 1977 - P.L. 95-217 and Federal Insecticide, Fungicide, and Rodenticide Act Amendments of 1975 - P.L. 94-140) point out the need for rapid and efficient means to predict chronically safe levels of many potential toxicants.

Monitoring of toxic wastes can take three general forms: physical, chemical, and biological. Fluctuations in the assimilative capacity

of a receiving stream occur daily and even hourly. In addition, interactions between chemicals may alter the actual toxicity to a community of organisms. These two points limit the value of using only chemical and/or physical monitoring. Individual organisms serve as useful integrators over the range of environmental variables in an effluent (in-plant) or when used in-stream. However, determination of the specific cause of a biological effect requires physical and/or chemical information so that all three are necessarily monitored.

The toxicity of chemicals in the aquatic environment is usually based upon the responses of fish. Acute and chronic fish bioassays are the standard methods of measuring toxicity. The chronic bioassay that measures the effects of survival, reproduction, and growth is the most widely used method for estimating long-term no-effect concentrations (Stephan and Mount 1973). This method is the most sensitive but it requires at least 1 year (with salmonid fish) to complete under the most favorable circumstances.

Intensive and extensive testing is required to reduce the risk of introducing dangerous pollutants into the environment. Conversely, overly-protective standards accomplish little ecologically while placing a financial burden on the public sector. A scanning tool for rapid, reliable prediction of safe toxicant levels is urgently needed (Maki 1979).

B. Research Objectives

This investigation attempts to determine no-effect levels of zinc for steelhead trout (Salmo gairdneri) based on short-term toxicity tests which could be used in new product screening, water quality criteria determination, and effluent biomonitoring by 1) developing an exposure system to expose steelhead to zinc solutions in flow-through chambers; 2) measuring respiratory patterns with external electrodes; 3) measuring two blood parameters after 96 hour exposures; 4) evaluating the experimental system under different regimes of temperature, hardness, and steelhead strain; and 5) comparing effect levels based on these short-term tests with maximum acceptable toxicant concentration (MATC) values determined in embryo-early juvenile exposures.

II. LITERATURE REVIEW

A. Bioassay Methods Currently Used

Fish bioassays are the standard method of measuring aquatic toxicity. Fish serve as sensitive indicators of water quality for many desirable uses, including human drinking water (Morgan 1978). Methods have been reviewed by Sprague (1969, 1970, 1971, 1973, and 1976) and Brungs (1973). An acute bioassay measures the concentration of a toxicant which is lethal to a certain percentage of fish (usually 50%) in a certain length of time (usually 96 hours). These tests, using death as the endpoint, require much interpretation and arbitrary extrapolation to determine biologically safe concentrations. In the past, a portion of the LC50 concentration (usually 0.1) was used as an estimate of the safe level of toxicant (Hart et al 1945; Henderson and Tarzwell 1957).

Chronic tests that measure the effects on survival, reproduction, and growth are the most widely used methods for estimating long-term no-effect concentrations (Stephan and Mount 1973). Mount and Stephan (1967) proposed that the maximum acceptable toxicant concentration (MATC) be defined as the highest toxicant concentration which does not adversely affect growth or reproduction during long-term chronic tests, usually full life-cycle exposures. This is currently the most accurate method for predicting safe concentrations and thus developing water quality criteria (National Academy of Sciences and National Academy of Engineering 1973), if the species tested is the most sensitive one in the ecosystem. The MATC divided by the 96 hr LC50 produces an application factor (AF) which is used to determine safe concentrations for other species and other receiving waters based upon their LC50's. However, Mount (1977) concluded that due to problems in the determination of AF's, this approach has questionable utility because the method is not supported by the data base. He further stated that no more accuracy is gained by using an AF and an LC50 than by simply selecting an MATC and using that value for all fish species. The environmental clearance of

large numbers of chemicals by chronic testing is expensive and time consuming. Alternatives to the standard fish bioassays need to be examined.

The embryo-larval and early juvenile life stages are the most or among the most sensitive periods of a fish's life. McKim (1977) examined data collected from 1969 to 1976 involving 56 life-cycle toxicity tests with 34 chemicals and four species of fish. Eighty-two percent of the time the MATC estimated by early life stage exposures (30 days minimum post-hatch) was identical to that estimated by full or partial life-cycle exposures. In all 56 tests the MATC could be estimated within a factor of two by early life stage testing. Macek and Sleight (1977) concluded that, for a great majority of toxicants, the level which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration and that the most sensitive stages are embryos and fry. Eaton (1973) stated that these exposures are better than 96-h acute tests or other short-term tests. However, with many species, embryo-larval and/or early juvenile exposures require at least 3 months and are not really "short-term" tests.

Many types of short-cut methods are being evaluated for predicting safe levels of toxicants (Sprague 1976). Various behavioral and physiological aberrations are used to indicate stress due to the presence of toxicants. These include observations of several types of behavior such as degree of locomotor activity and orientation in a current (Drummond et al 1973; Warner et al 1966; and Kleerekoper 1976), feeding behavior (Drummond et al 1973; Mason and Davis 1976; Foster et al 1966; Cairns and Loos 1967; and Ludwig and Warner 1964), avoidance behavior (Davis 1976; Sprague and Drury 1969; and Sprague 1964, 1968), reduced spawning behavior, and alarm response behavior (Servizi et al 1969). Physiological change is the basis for many sublethal toxicity tests. Some of these end-points are metabolic rate, usually measured as oxygen consumed or carbon dioxide respired (Davis 1973; Cairns 1966; Cairns and Scheier 1964; Fry and Hart 1948; MacLeod and Smith 1966; and Eaton 1973), and a related test, the sealed jar residual oxygen bioassay (Carter 1962;

McLeay and Howard 1977; and McLeay 1976), olfactory sensitivity (Bardach et al 1965 and Hara et al 1976), temperature tolerance (Howard 1973; Howard and Walden 1974; and McLeay and Howard 1977), swimming speed or ability (Howard 1973; Fry and Hart 1948; McLeay and Howard 1977; MacLeod and Smith 1966; Sprague 1971; and Bull and McInerney 1974), salt water tolerance (Schreck and Lorz 1978; Lorz et al 1978; and Lorz and McPherson 1977), and tissue toxicant residues (Mount 1964; Mount et al 1966). Several other types of sublethal toxicity tests, directly related to my research, deserve special attention.

Embryonic development and/or short-term growth, which are components of both embryo-larval and life-cycle toxicity tests, are used as short-term sublethal estimates although the time required is usually longer than one week (Davis 1976; Ellis 1967; Tokar and Owens 1968; Brungs 1969; Skidmore 1964; Mason and Davis 1976; Pickering and Vigor 1965; Servizi et al 1966; and McLeay and Brown 1974).

B. Blood Parameters as Indicators of Sublethal Toxicity

Various blood parameters have been used as measures of sublethal stress. Some investigators have used changes in electrophoretic patterns of serum protein as an estimate of sublethal toxicity (Fujiya 1961; Bouck and Ball 1965; and Steward and Li 1969). Ionic and cholesterol concentrations have been used as sublethal endpoints (Eisler and Edmunds 1966; McKim et al 1970), as have blood glucose levels (McLeay 1977; McLeay and Howard 1977; Nakano and Tomlinson 1967; McLeay 1973; and McLeay and Brown 1974), catecholamine concentration (Nakano and Tomlinson 1967), and serum cortisol levels (Schreck and Lorz 1978; Donaldson and Dye 1974; and Lorz et al 1978). Changes of enzyme activity levels have been measured in blood and tissues and used as sublethal responses. Acetylcholinesterase inhibition is a common enzyme assay used to detect organophosphate insecticides, but there are problems with applying the results of this assay (Gibson et al 1969). Using glutamicoxalacetic

transaminase, Bell(1968) identified salmon exposed to several toxicants. Other enzymes examined in fish are ornithine aminotransferase (Brown 1976; Wekell and Brown 1973), glutamine synthetase (Webb and Brown 1976), cytochrome oxidase (Pederson et al 1974) lactate dehydrogenase, glutamate dehydrogenase, creatine phosphokinase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, glucose-6-phosphatase, and glutathione reductase (Racicot et al 1975).

Routine hematological techniques such as determination of hematocrit, hemoglobin, and white and red blood cell counts have been useful in assessing the health of fish (Blaxhall 1972; Hickey 1976) and in detecting sublethal stress (Soivio and Oikari 1976; McKim et al 1970; Bills and Hunn 1976; and Iwama et al 1976). Stressors were shown to cause leucopenia in salmonid fishes (Weinreb 1958; McLeay 1970, 1973; and McLeay and Gordon 1977). Decreases in hematocrit values of fish receiving chronic toxicant exposures have been observed (Warner 1967; McLeay 1973) and hematocrit increases have been seen in fish subjected to acute stress (Swift and Lloyd 1974; McLeay and Gordon 1977). Sprague (1971) indicated that the variable response of hematocrit makes it a less sensitive test than survival, growth, or other endpoints. A decrease in numbers of circulating lymphocytes may be a more reliable indicator of stress than any other hematological parameter (Belova 1966). Hickey (1976) concluded that white blood cell counts may be the most definitive test in determining physiological responses of fish to toxic substances. The leucocrit test of McLeay and Gordon (1977) is based on the determination of the volume of packed leucocytes plus thrombocytes expressed as a percentage of the whole blood. Values for leucocrit and actual white blood cell counts were highly correlated (95.3 % of the contents of the "buffy" layer was composed of leucocytes and thrombocytes).

C. Respiratory Monitoring to Detect Sublethal Toxicity

In 1929, Belding was the first to use fish respiration as an indicator of a toxic environment by demonstrating that hydrogen

sulfide increased ventilatory frequency (Belding 1929). Subsequently, this approach to sublethal toxicity was neglected until about 1970. Sprague (1971) stated that respiratory impairment in fish is related to clear-cut effects of toxicant stress. Respiratory activity changes have been used by several investigators as indicators of an organism's response to toxicant stress (Drummond et al 1973; McPherson 1973; Lunn et al 1976; Maki et al 1973; Hargis 1976; Morgan and Kuhn 1974; Schaumburg et al 1967; Cairns et al 1970; and Sparks et al 1972a, 1972b). The respiratory movements measured represent muscular expansion and contraction of the buccal and opercular cavities which drive water over the gill surfaces. This mechanism and its nervous control were described by Hughes (1970), Hughes and Shelton (1957, 1958, and 1962), and Ballintijn and Hughes (1965). Examination of ventilatory movements represents an indirect means of assessing oxygen consumption (van der Schalie 1977). The amount of oxygen removed from water depends upon the area of the gills and the rate of water movement over the gills.

Several methods have been used to monitor ventilatory movements of fish (Heath 1972). Some investigators have directly observed the fish (McPherson 1973; Hargis 1976) while others have recorded water pressure changes in the buccal and/or opercular cavities associated with ventilatory activity. Using this method, a tube is placed adjacent to these cavities (Hughes and Shelton 1958) or a cannula is inserted in the fish (Saunders 1961). Heath (1972) concluded that this technique provides the most information about respiratory responses. Sensitive pressure transducers measure changes in water pressure and the resultant signals are displayed on an oscilloscope or recorded on a strip-chart recorder. With slight modification, many investigators have used this method (Schaumburg et al 1967; Hughes and Saunders 1970; Hughes and Roberts 1970; Young 1972; Sparks et al 1972a, 1972b; Davis 1973; Sellers et al 1975; and Bimber et al 1976). With the third method, muscle action potentials associated with opening and closing of the buccal cavity, branchial arches, and operculum, rather than water pressure changes, are measured with external electrodes (Camougis 1960; Goodman and

Weinberger 1971; and Heath 1972), amplified, and recorded. This technique, using an electrode chamber (Spoor et al 1971), allows detection of ventilatory activity without the stress caused by implanting catheters (Drummond and Carlson 1977).

The primary bits of information which can be derived from the ventilatory signal are the respiratory rate and the coughing rate. Ventilatory amplitude, indicative of the depth of breathing, can be inferred from the height of the signal and the amplifier sensitivity setting, but the orientation of the fish in the electrode chamber influences this parameter, i.e. distance from the electrodes determines the relative height of the peaks on a strip chart. Large, whole-body movements can obscure the complete ventilatory signal and direct contact of the fish with an electrode can result in perception of heart beat signals. Water displacement at the electrode surface was formerly believed to be the cause of the electric potential change (Goodman and Weinberger 1971; Spoor et al 1971). Heath (1972) refuted this by showing that respiratory muscle action potentials were the source of the change. Gruber et al (1978) used a cinematographic approach to demonstrate significant correlation between opercular movement and the change as recorded on a strip-chart recorder. Barham et al (1969) reported bioelectric potentials generated by muscles of between 0.01 and 40 microvolts. These small electric muscle action potentials must be amplified and filtered of noise before recording. Drummond and Dawson (1974) developed a simplified solid state amplifier to replace the expensive equipment previously used (Goodman and Weinberger 1971; Spoor et al 1971). Gruber et al (1977) designed an amplifier with built-in noise immunity. Interfacing the amplifier with a computer system allows continuous, automatic monitoring and rapid analyses of data collected (Cairns et al 1970; van der Schalie 1977; and Thompson et al 1978).

The cough reflex, or gill purge response (Drummond and Carlson 1977), is a momentary reversal of water flow over the gills normally used to clean particulate matter from the lamellae. Fish ordinarily exhibit a coughing response when unstressed and increase in frequency of the response is associated with gill irritation. Increased

coughing frequency indicates that a fish is undergoing a "loading stress" (Brett 1958) because the expenditure of energy required for maintenance is increased (MacLeod and Smith 1966).

Many materials affect ventilatory and/or coughing rates at sub-lethal toxicity levels including heavy metals (Sparks et al 1972a, 1972b; Sellers et al 1975; McIntosh and Bishop 1976; Morgan 1977; Cairns et al 1973b; Drummond et al 1974; Sprague 1964; Drummond et al 1973; Dandy 1967; Martens et al 1970; and McPherson 1973), complex effluents (Walden et al 1970; Davis 1973; Howard and Walden 1974; Schaumburg et al 1967; Thomas and Rice 1975; Carlson and Drummond 1978; and Maki 1979), pesticides (Morgan 1977; Lunn et al 1976; Schaumburg et al 1967; Bull and McInerney 1974; and Bimber et al 1976), suspensions of pulpwood fibers (MacLeod and Smith 1966), chlorine and sulfides (Bass 1975; Dandy 1972; and Servizi et al 1969), and other materials such as coal dust, wood pulp, kaolin, ammonia, and alkylbenzene sulfonate (Hughes 1975). Various environmental parameters also change ventilatory patterns including dissolved oxygen (Hughes and Saunders 1970), temperature (Hughes and Roberts 1970), pH (Hargis 1976), activity level (Heath 1973; Sutterlin 1969), turbidity (Horkel and Pearson 1976), and season of the year (Beamish 1964).

Heavy metal salts are known to cause respiratory aberrations in fish. Sparks et al (1972a), measuring cough frequency, found the response to sublethal zinc exposure to be rapid and proportional to the concentration from two mg/l upward. Sparks et al (1972b) found that bluegills (Lepomis macrochirus) increased breathing rates or rate variances when exposed to concentrations from 2.55 to 8.7 mg/l of zinc. However, these concentrations are at least ten times the safe level determined by spawning inhibition and fry mortality. Cairns et al (1973a) showed that abnormal ventilatory frequency can be observed well before irreversible damage occurs in bluegills exposed to zinc and that they respond to sublethal concentrations (of two to three mg/l). Sellers et al (1975) reported changes in either ventilation rates, coughing rates, or pressure amplitude levels when rainbow trout (Salmo gairdneri) were exposed to

concentrations at or below the 48-h LC50 for copper and zinc. The cough response was proclaimed as a more significant and rapid indicator of toxicity than ventilatory rate (Drummond and Carlson 1977; Sprague 1971). Coughing frequency was shown to increase at or slightly below safe concentrations of copper (Drummond et al 1973) and mercury (Drummond et al 1974) for brook trout (Salvelinus fontinalis). However, Maki (1979) urges use of ventilatory frequency because it is much easier to quantify than cough frequency. Identification of a cough event is subjective, with large variations between counts made by different personnel.

Not all fish readily lend themselves to testing in an electrode chamber (King 1978). Bluegills (Sparks et al 1972a), brook trout (Drummond et al 1973), rainbow trout (Sellers et al 1975), largemouth bass (Micropterus salmoides) (Morgan and Kuhn 1974), and fathead minnows (Pimephales promelas) (King 1978) have all been tested for respiratory aberrations but steelhead trout have not. Ventilatory signals vary in form in different fish. Bluegills exhibit a typical bimodal pattern while fathead minnows and goldfish (Carassius auratus) show irregular patterns in which body movements frequently obscure the respiratory signal (King 1978). These actions make determination of cough frequency even more difficult.

D. Zinc as an Aquatic Toxicant

Zinc was the toxicant of choice in this study because of its ubiquitous nature and known toxicity to fish. Zinc is known to be an essential dietary requirement for rainbow trout and an adequate ration is between 15 and 30 ppm of the food (Ogino and Yang 1978). These authors reported zinc deficiency to be manifested as cataracts, poor protein digestion, and erosion of the integument. However, aqueous zinc at high levels is known to be harmful to fish. Hiltibran (1971) suggested that inhibition of oxygen uptake within cells, thereby altering intracellular metabolism of oxygen and phosphate, is more important than external functions such as mucus precipitation. Cradall and Goodnight (1963) noted degenerative histological changes,

growth inhibition, and retarded sexual maturation and concluded that secondary effects such as stress and lack of nutrition were the most important responses. These investigators used guppies (Poecilia reticulata) exposed to sublethal zinc concentrations (1.15 mg/l). The presently accepted opinion regarding chronic or sublethal effects of zinc is that damage to gill epithelial tissue, accompanied by detachment of this tissue layer, leads to an increased diffusion distance between water and blood (Lloyd 1960; Jones 1964; Skidmore 1970; and Skidmore and Tovell 1972). If increased ventilatory activity cannot overcome this deficit, arterial and then tissue hypoxia result. Sellers et al (1975) noted that mucus accumulation on the gills contributes to hypoxia which substantiates earlier opinions (Carpenter 1927, 1930; Dilling et al 1926; Jones 1938, 1939; Ellis 1937; and Westfall 1945) that coagulation and/or precipitation of mucus on the gills leads to suffocation.

III. METHODS AND MATERIALS

A. Test Design

The value of short-term tests (respiratory responses, hematocrit, and leucocrit) depends upon a direct correlation of the results to MATC estimates. An MATC value was determined by employing exposure to zinc of steelhead trout from eggs to early juveniles. Ninety-six hr exposures were then conducted on steelhead smolts and monitored for ventilatory activity both before and during exposures and also monitored for leucocrit and hematocrit after the exposures. Response levels determined from the short-term tests were compared to MATC levels and published LC50 acute values to evaluate the short-term tests as predictors of chronically safe zinc concentrations. Ten separate short-term tests were run which covered three temperature values, two hardness values, and two strains of steelhead trout (Table 1).

B. Test Fish

Fertilized steelhead eggs were obtained from Oregon Department of Fish and Wildlife hatcheries on the day of spawning. One group of fish was spawned on the Alsea River in February 1977 and the other group was spawned on the North Santiam River in May 1978. All eggs were dipped 10 minutes in disinfectant (Wescodyne) solutions of 1:150 dilution, which had been adjusted to pH 7 with sodium bicarbonate, before being incubated at Western Fish Toxicology Station. Eggs used in embro-larval exposures were immediately placed in upwelling incubators built for these tests. All other eggs were placed in vertical-flow Heath-Techna incubators.

Fish reaching the swim-up stage were transferred to troughs and later to larger tanks. Fish of swim-up stage and older were fed Oregon Moist food, but test fish were not fed for 48 hrs prior to or during short-term tests. Fish in the embryo-early juvenile exposure were fed from one to four times per day at a rate of 5.5% of the body

TABLE 1. NOMINAL TEST CONDITIONS FOR SHORT-TERM TESTS AND DATES COMPLETED

| TEST | TEMPERATURE (C) | HARDNESS (mg/1) | STEELHEAD STRAIN | DATE COMPLETED |
|------|--------------------|--------------------|---------------------|-------------------|
| 1 | 12 | 25 | Aalsea R. | 3/25/79 |
| 2 | 12 | 25 | Aalsea R. | 4/23/79 |
| 3 | 12 | 25 | Aalsea R. | 5/7/79 |
| 4 | 7 | 25 | Aalsea R. | 6/4/79 |
| 5 | 17 | 25 | Aalsea R. | 6/17/79 |
| 6 | 12 | 125 | Aalsea R. | 7/17/79 |
| 7 | 12 | 125 | Aalsea R. | 7/31/79 |
| 8 | 12 | 125 | Aalsea R. | 8/10/79 |
| 9 | 12 | 25 | Santiam R. | 9/28/79 |
| 10 | 12 | 25 | Santiam R. | 10/13/79 |

weight. Frequency of feeding decreased from 7 to 5 days per week after 14 days of exposure.

Mean fork lengths of steelhead in the short-term tests ranged from 16.4 to 25.0 cm (Table II). Other gross parameters were: mean wet weight - 53.0 to 140.2 g and mean condition factor - 8.194 to 12.067. The wet weight of the survivors in the embryo-early juvenile test averaged 0.585 g based on batch weights of 1,147 fish.

C. Dilution Water

Laboratory dilution water was supplied by wells on the banks of the nearby Willamette River. Well water quality was essentially that of the river except for lower concentrations of suspended solids, organic carbon, iron, and manganese (Chapman 1978b). Hardness, alkalinity, and dissolved solids increased several-fold during winter months in well-water. Samuelson (1976) presented a thorough analysis of well and Willamette River water quality chemistry. Water for all tests was pretreated by ultraviolet sterilization (Aquafine Electronic Liquid Sterilizer Model C4S-2), aerated to near air saturation levels by jetting and spraying, and adjusted for hardness and constant temperature. Dissolved oxygen, pH, total alkalinity, and total hardness were measured daily from at least one aquarium containing fish by methods of the American Public Health Association et al (1971) and the U.S. Environmental Protection Agency (1974). These determinations were made at least once per week for each metal concentration. Water temperature was recorded continuously during all tests with a Taylor recorder (Taylor Instrument Co. Serial No. B76JM117 3263). Mean temperature was determined by averaging readings taken from the recorder chart every 3 hours.

Water quality was relatively constant within tests (Table III). Test temperatures were stable with standard deviations never exceeding 0.9 C and were always within 1.0 C of nominal temperatures (compare with Table I). Total hardness and alkalinity were positively correlated and showed low variability between tests. In the low hardness tests (embryo-early juvenile test and tests 1-5, 9, and 10),

TABLE II. FISH SIZE IN SHORT-TERM TOXICITY TESTS (N = 16 except where noted)

| TEST | WET WEIGHT + SD (g) | FORK LENGTH + SD (cm) | CONDITION FACTOR + SD $K = (W/L^3) \times 1000$ |
|----------------|---------------------------|-----------------------------|---|
| 1 | 111.4 (+18.6) | 22.6 (+1.3) | 9.578 (+0.507) |
| 2 ^a | 140.2 (+34.4) | 25.0 (+2.1) | 8.895 (+0.536) |
| 3 ^a | 108.4 (+26.9) | 22.8 (+1.8) | 8.890 (+0.569) |
| 4 ^b | 123.3 (+18.1) | 24.2 (+1.2) | 8.860 (+0.526) |
| 5 ^c | 117.6 (+12.5) | 24.2 (+1.1) | 8.194 (+0.652) |
| 6 | 128.7 (+22.6) | 24.9 (+1.2) | 8.252 (+0.710) |
| 7 ^c | 127.0 (+16.0) | 24.3 (+1.0) | 8.881 (+0.832) |
| 8 | 122.5 (+20.9) | 24.1 (+1.5) | 8.696 (+0.911) |
| 9 ^c | 58.0 (+11.8) | 17.2 (+1.3) | 11.640 (+0.486) |
| 10 | 53.0 (+9.0) | 16.4 (+1.0) | 12.067 (+0.745) |

^aWeights and K values exclude two fish dead prior to sampling

^bWeights, lengths, and K values exclude two fish dead prior to sampling

^cWeights and K values exclude one fish dead prior to sampling

TABLE III. MEASURED WATER QUALITY PARAMETERS

| TEST | TEMPERATURE ^a (C) | TOTAL HARDNESS ^a (mg/l) | TOTAL ALKALINITY ^a (mg/l) | pH ^b | DISSOLVED OXYGEN ^c (mg/l) |
|------------------|---------------------------------|--|--|------------------------|--|
| E-L ^d | 12.7 (±0.4) 55 | 27 (±4) 51 | 24 (±3) 51 | 7.1 (6.7-7.4) 47 | 10.0 (±0.4) 47 (93%) |
| 1 | 12.0 (±0.2) 55 | 27 (±3) 9 | 27 (±5) 7 | 6.9 (6.8-7.0) 7 | 9.6 (±0.4) 7 (89%) |
| 2 | 12.1 (±0.3) 41 | 23 (±1) 5 | 25 (±1) 5 | 7.0 (6.9-7.1) 5 | 9.7 (±0.1) 5 (90%) |
| 3 | 11.8 (±0.2) 35 | 25 (±5) 8 | 26 (±3) 7 | 7.1 (6.9-7.1) 8 | 9.8 (±0.1) 8 (91%) |
| 4 | 8.0 (±0.3) 42 | 26 (±1) 8 | 25 (±1) 8 | 7.1 (7.0-7.2) 8 | 10.9 (±0.3) 8 (92%) |
| 5 | 17.2 (±0.2) 41 | 26 (±1) 7 | 24 (±1) 7 | 7.1 (7.0-7.3) 7 | 8.8 (±0.3) 7 (91%) |
| 6 | 12.5 (±0.9) 40 | 124 (±7) 6 | 112 (±5) 6 | 7.1 (6.7-7.3) 6 | 9.6 (±0.8) 6 (90%) |
| 7 | 12.3 (±0.7) 42 | 108 (±28) 9 | 120 (±37) 9 | 7.1 (6.9-9.7) 9 | 9.8 (±0.4) 9 (92%) |
| 8 | 12.6 (±0.9) 40 | 119 (±4) 8 | 114 (±3) 8 | 7.2 (7.1-7.2) 8 | 10.0 (±0.3) 8 (93%) |
| 9 | 12.1 (±0.1) 37 | 22 (±1) 8 | 24 (±1) 8 | 7.2 (6.8-7.3) 8 | 10.0 (±0.3) 8 (92%) |
| 10 | 12.0 (±0.1) 42 | 22 (±1) 6 | 24 (±1) 6 | 7.1 (7.0-7.2) 6 | 10.3 (±0.4) 6 (95%) |

^aTop line: Mean; Bottom line: (±SD) N

^bTop line: Median; Second line: Range; Bottom line: N

^cTop line: Mean (±SD); Bottom line: N (% Saturation)

^dEmbryo-Larval Test

measured values were always within 3 mg/l of nominal concentrations. In the high hardness tests (tests 6-8), measured values were within 17 mg/l of nominal concentrations (compare with Table I). Dissolved oxygen exceeded 89 % of saturation in all tests and averaged 92 %. Median pH values ranged from 6.9 to 7.2 among the tests and showed ranges covering an average of 0.5 unit.

D. Toxicant

Zinc was added to the water in the form of analytical reagent grade zinc chloride (Mallinkrodt, Inc.) although exposure concentrations are expressed as Zn^{++} ion. Stock solutions were prepared from the zinc salt and were acidified with 0.1 ml of concentrated nitric acid per liter of stock solution. Toxicant concentrations were chosen on the basis of comparison with available bioassay data (Andrew et al in review; Chapman 1978a, 1978b; Spehar et al 1978; Benoit and Holcombe 1978; Chapman and Stevens 1978; and Brown 1968). Water samples for toxicant analyses were taken daily from one tank containing each zinc concentration. Zinc samples were collected in acid-washed, distilled water rinsed containers, and the samples were acidified by the addition of 0.1 volume percent of concentrated nitric acid. Zinc levels were measured by flame atomic absorption spectrophotometry (Perkin-Elmer Model 403). Absorption was linear within the working range and analytical precision was ± 3 $\mu\text{g/l}$.

Tests 1-3 were nominal replicates. Average measured concentrations were as follows: 5, 41, 81, and 146 $\mu\text{g/l}$ Zn. Measured concentrations decreased from test 1 to test 3 because of problems with the dilution apparatus. Tests 6-8 were also replicates with average concentrations as follows: <5, 220, 433, and 803 $\mu\text{g/l}$ Zn. Tests 9 and 10 were replicates and had average concentrations of <5, 47, 103, and 190 $\mu\text{g/l}$ Zn (Table IV).

TABLE IV. MEASURED ZINC CONCENTRATIOND ($\mu\text{g}/1$) IN EMBRYO-LARVAL AND SHORT-TERM EXPOSURES

| TEST | MEAN | STANDARD DEVIATION | N | TEST | MEAN | STANDARD DEVIATION | N |
|---------------|------|--------------------|-----|------|------|--------------------|----|
| Embryo-larval | <5 | a | 9 | 6 | <10 | a | 4 |
| | 86 | 6 | 17 | | 224 | 29 | 4 |
| | 148 | 11 | 12 | | 441 | 44 | 4 |
| | 262 | 20 | 12 | | 814 | 86 | 4 |
| | 444 | 30 | 13 | | 7 | < 5 | a |
| 819 | 50 | 17 | 224 | 9 | | 5 | |
| 1 | 11 | 5 | 6 | 440 | | 15 | 5 |
| | 51 | 9 | 6 | 814 | | 24 | 5 |
| | 94 | 16 | 6 | 8 | | < 5 | a |
| | 176 | 25 | 6 | | 212 | 27 | 5 |
| | 2 | 2 | 2 | | 4 | 419 | 37 |
| 34 | | 6 | 4 | | 781 | 66 | 5 |
| 73 | | 5 | 4 | | 9 | < 2 | a |
| 144 | | 21 | 4 | 52 | | 3 | 5 |
| 3 | | 3 | 2 | 4 | | 115 | 10 |
| | 38 | 4 | 4 | 200 | | 8 | 5 |
| | 76 | 11 | 4 | 10 | | < 5 | a |
| | 117 | 5 | 4 | | 42 | 9 | 5 |
| | 4 | < 5 | a | | 5 | 91 | 10 |
| 41 | | 5 | 5 | | 179 | 22 | 5 |
| 78 | | 4 | 5 | | | | |
| 147 | | 8 | 5 | | | | |
| 5 | | < 5 | a | 4 | | | |
| | 33 | 8 | 4 | | | | |
| | 72 | 10 | 4 | | | | |
| | 141 | 16 | 4 | | | | |

a Standard deviations could not be computed for these concentrations because chemical analyses were near the detection limit and values were given as less than five $\mu\text{g}/1$.

E: Embryo-Early Juvenile Exposure

The early life history test was conducted with Alsea River steelhead at a nominal total hardness of 25 mg/l as CaCO_3 , as determined by the EDTA titrimetric method (APHA et al 1971). Temperature was maintained at a nominal level of 12 C by first chilling with two 825 ft-lbs sec^{-1} coolers (Dunham-Bush Inc. Model SCCP) and then heating through Honeywell unit (Honeywell Industrial Div. Electronik 111) -mediated tubular heating elements (General Electric). Hardness was controlled by mixing ambient well water with the appropriate amount of reverse-osmosis (RO) -treated well water. The RO unit was manufactured by Purification Techniques Inc. (Model P803). Uniform water quality was necessary because variations affect zinc toxicity and negate comparisons among tests. Tests were conducted in 19 l glass aquaria with a water volume of approximately 14 l. Aquaria were dosed by a proportional diluter similar to that described by Mount and Brungs (1967) and delivered 2.15 l/concentration/cycle. The diluter provided six zinc concentrations, including the control, and the flow was split equally between duplicate aquaria. The 50 % replacement time for aquaria was 20 minutes. Calculations were based on the method of Sprague (1969).

The test system was shrouded in black plastic, both outside the system and between adjacent aquaria, to control light and to lessen disturbance. Photoperiod was adjusted weekly to correspond to the existing local day length. Thirty minute dawn and dusk periods were provided by two 25-watt incandescent lamps powered through a motor-driven rheostat. Daytime illumination was supplied by two 40-watt fluorescent tubes and light intensity at the water surface was approximately 500 lux.

Prior to the test, following disinfection of the eggs, equal numbers of eggs were randomly assigned to each of the 12 aquaria by volume of a 13 mm x 16 cm glass tube. About 140 eggs were placed in incubators made of 23 cm lengths of 10.2 cm PVC pipe with glass bottoms. Each was filled to a depth of 12.7 cm with three layers of marbles, of decreasing diameter going up the incubator, to simulate

streambed gravel. Eggs were placed on top of the 'gravel'. Water entered the bottom of the incubators through a 1.9 cm PVC pipe, flowed up through the marbles, over the eggs, exited through perforations near the tops of the 10.2 cm pipes, and drained out through the aquaria's standpipe drain (Figure 1).

Toxicant was introduced 1 day after placing the eggs in the incubators and continued for 72 days. Fish were exposed for 47 days after hatching. Unfertilized eggs were removed daily. Dead eggs were gently removed and preserved in Stockard's solution. At the eyed stage, eggs were shocked by vigorous turbulence from a hose carrying well water for about 7 seconds. Within 3 days after shocking all eggs were hatched and the remaining dead embryos were removed. At the time of swim-up, sac-fry were liberated into the aquaria proper which had screens over the standpipes to prevent loss of fry. At this time and once weekly thereafter six control fish were killed with a rapidly lethal dose of MS-222, blotted dry, yolk sacs were excised, and the fish were weighed. By counting survivors in each aquaria, a weekly ration of food was calculated based on 5.5 % of body weight per day. Loading factors were constantly changing due to death and growth but never exceeded 4.5 g/l of aquarium water or 0.05 g/l/day based on flow rates. These loading factors were well within recommended limits for flow-through toxicity tests (Committee on Methods for Toxicity Tests with Aquatic Organisms 1975). The test was initiated by allowing the zinc concentrations in the tanks to increase at the rate determined by volume replacement times; nominal concentrations were approached in less than 2 hours. Checks for dead fry were made at least once daily after swim-up; dead fish were removed when observed. Criteria of death were absence of ventilation, loss of irritability, and absence of muscular motion. Survival was used as the endpoint for determining the MATC range. Surviving fish were killed, blotted dry, and weighed at the termination of the exposure period. Mean weights were obtained for each concentration group.

Figure 1. Diagram of incubator used in the embryo-early juvenile test.

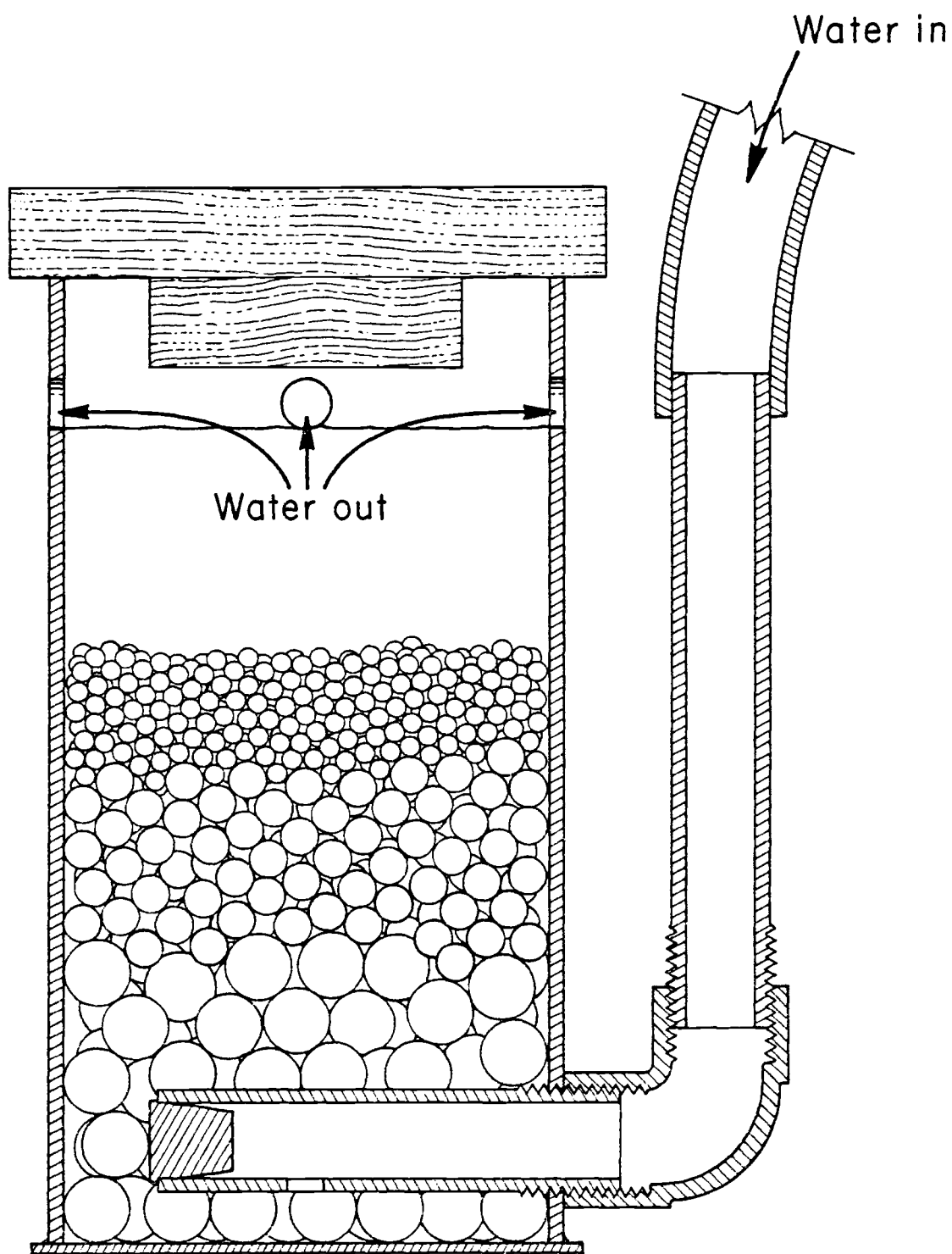


Figure 1.

F. Short-Term Tests for Respiratory and Hematological Response

The design for short-term exposures was as follows: an acclimation period of between 3 and 8 days, followed by a 24-h pre-exposure period, followed by a 96-h zinc exposure period. Tests in electrode chambers similar to those described by Spoor et al (1971) were done to determine a range of zinc concentrations which the short-term tests are capable of detecting. Each test included 16 steelhead trout, four in each concentration, including the controls. Water and zinc solutions were delivered by glass tubes from a continuous-flow serial diluter similar to that described by Garton (1980). A diaphragm pump (Control Products Corp. Chemical Feed Pump Model 12721-11) delivered the zinc solution to a toxicant head box. Subsequent mixing chambers allowed for the combination of dilution water and toxicant to obtain the final concentrations. The flow rate into each electrode chamber was 200 ± 6 ml/min. Chambers measured $38.1 \times 15.2 \times 15.2$ cm and the compartments in which the fish were confined were only 27.9 cm long (Figure 2). The water volume of each chamber was 6.64 l, resulting in a 95 % replacement time of 100 minutes (Sprague 1969). All chambers were fitted with white plexiglass lids and were painted white on the out-sides and front to reduce disturbance. The backs of the chambers were left unpainted. The entire system of chambers and water delivery apparatus was shrouded with a black plastic curtain. Photoperiod control was maintained by a double bank of 40-watt fluorescent tubes turned on and off by an electric time switch (Dayton Electric Mfg. Co. Model 2E026) to correspond to local day length. In addition, room lights were left on outside the curtain constantly and some light always illuminated the testing compartment. Illumination at the water surface was 165 ± 48 lux under the chamber lids although the fish could observe more light because of the unpainted backs of the chambers.

Temperature control was achieved by first cooling ambient water with two Dunham-Bush chillers (Models NSPC 20HQ and NSPC 25H) and then heating with two General Electric tubular heating units placed in the aeration tank. For test four, additional cooling of

Figure 2. Diagram of electrode chamber used in the short-term zinc exposures.

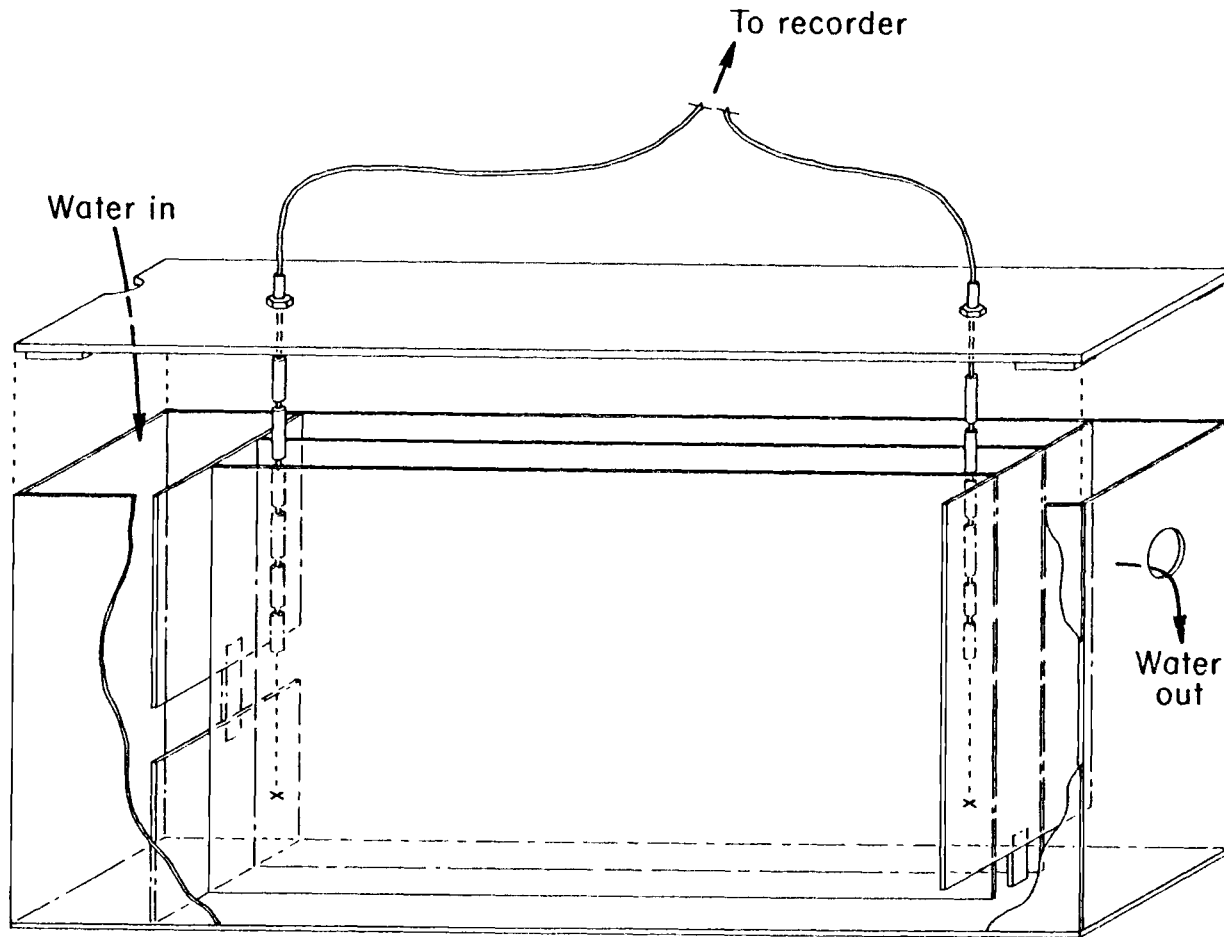


Figure 2.

dilution water was attained by immersing 1.3 cm steel heat exchange coils in a large tank containing 'super-chilled' water of 4 C. Dilution water flowed through these coils and into the diluter.

Low hardness regimes (25 ppm) were achieved with an RO unit as in the embryo-larval test. High hardness regimes (125 ppm) were maintained with a hardness generator, consisting of a large steel tank filled with limestone (Figure 3). Ambient well water, saturated with CO₂, flowed through the tank and was aerated to neutralize the pH.

Fish were transferred from holding tanks to the electrode chambers under anesthesia by 50 mg/l MS-22. Randomly selected fish were gently placed in individual chambers receiving dilution water and the lids were taped to the chambers. Ambient temperature of the holding tank water increased during the course of the study from 8.5 to 18.0 C. However, chamber temperatures were always within 2 C of ambient temperatures when the fish were put into the chambers. These temperatures were gradually adjusted to the test temperatures and no rate of change greater than 2.5 C in any 24 hr period (range from 0 to 2.5 C/day) was used. The short-term testing system is schematically diagrammed in Figure 4.

G. Respiratory Monitoring

The system for ventilatory monitoring included pairs of stainless steel electrodes (one each positive and negative) which were partially covered by Tygon tubing to reduce physical contact by the fish. These electrodes were fitted into pin jack connectors in the chamber lids (Figure 5) and connected by microphone cables to a multiplex switching device (Figure 6). This solid state device relayed muscle action potentials from the chambers to a four-channel strip chart recorder (Beckman Instruments, Inc. Model R-511A).

The recorder contained pre-amplifiers, amplifiers, rectilinear servo-pens, and a strip chart drive. The multiplexer was constructed so that any length of sampling period and any length of pausing between samples can be selected. A group of four fish was sampled

Figure 3. Hardness generator.

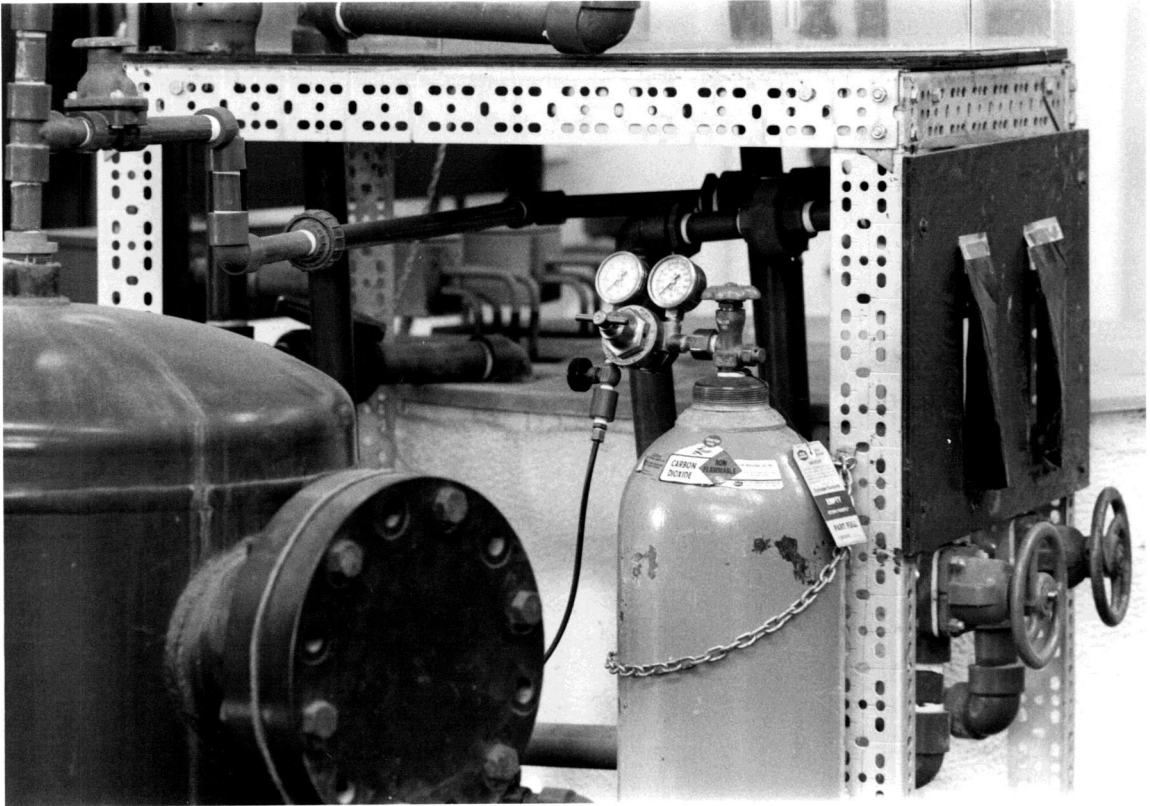


Figure 3.

Figure 4. Schematic diagram of the short-term testing system.

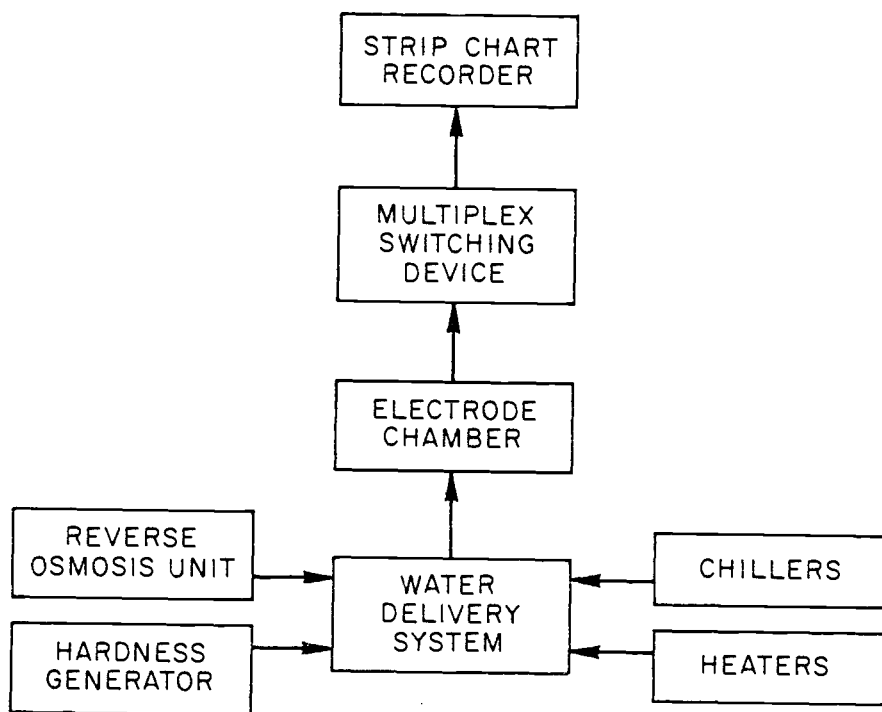


Figure 4.

Figure 5. Electrode chambers with cables leading to the recording apparatus.

Figure 6. Multiplex switching device.

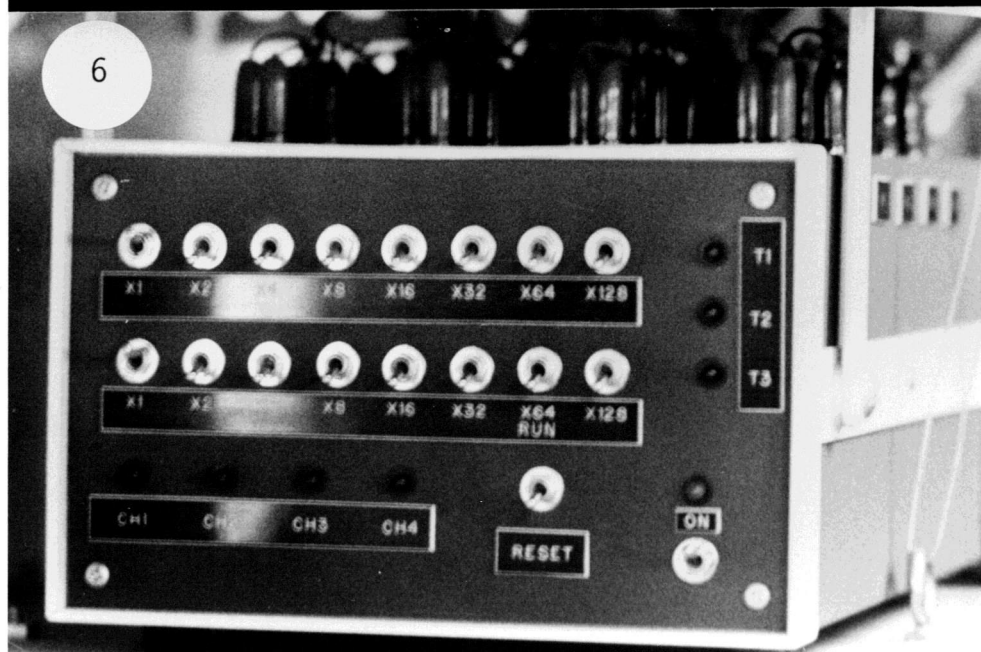
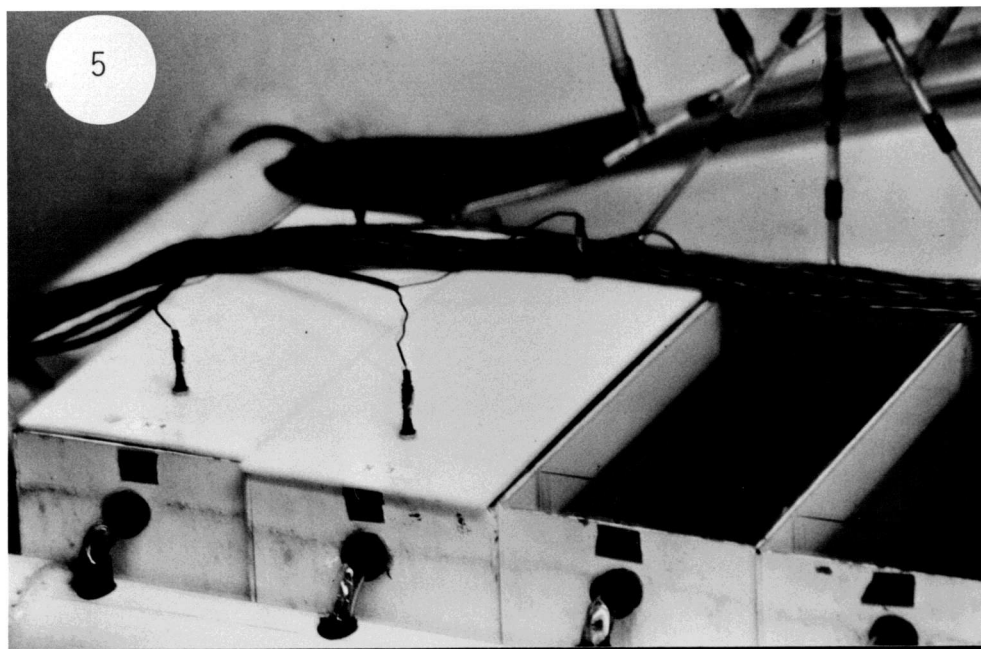


Figure 5.

Figure 6.

simultaneously after a 15 second start-up period. The pre-selected sampling period was then followed by another 15 second start-up period and the second group of four fish was then sampled. This sequence continued until all 16 fish were monitored. The pre-selected sampling period was always four minutes but the wait periods were varied. The multiplexer enabled sampling of 16 fish with a four-channel recorder. During the 24 hour control period, respiratory sampling was done every 2 hours. Immediately following this pre-exposure period, zinc solution was introduced into the system. From the initiation of exposure until 20 hours had elapsed, sampling was done every hour; from 20 to 50 hours, sampling was done every 2 hours; and from 50 hours until the termination of zinc exposure, sampling was done every 4 hours. Ventilatory frequency was manually counted on the strip chart records for 2 minutes at every sampling time for every fish during the 4 day exposure. Every fish was counted every 4 hours during the 24 hour control period. An original objective of the study was to examine coughing frequency. However, large discrepancies in identifying a cough event on the strip chart record occurred between personnel counting the same record. This involved highly subjective judgement so the respiratory research focused on ventilatory rates which could more objectively be enumerated.

H. Blood Parameters

Upon conclusion of the zinc exposures, all fish were deeply anesthetized and blood samples were withdrawn for analyses. In sequence, fish were individually, in a random manner, narcotized (100 mg/l MS-222), killed with a blow to the top of the head, and blood was removed by severing the caudal peduncle with a sharp scalpel. Heparinized capillary tubes (Van-Lab Catalog No. 15401-628) of 75 X 1.12-1.17 mm ID, were filled with blood and then centrifuged (International Equipment Co. Micro-Capillary Centrifuge Model MB) for nine minutes at 11,500 rpm. Tubes were centrifuged within 10 minutes after sampling and examined for leucocrit and hematocrit

values within 10 minutes after centrifugation. Hematocrit, or the volume of packed erythrocytes, was measured by the microhematocrit method of Hesser (1960) and Snieszko (1961), using a capillary reader (International Equipment Co. Model CR Micro-Capillary Reader), Leucocrit, or the volume of packed leucocytes plus thrombocytes expressed as a percentage of the whole blood column, was measured according to the method of McLeay and Gordon (1977). The height of the 'buffy' layer to the nearest 0.01 mm using a dissecting microscope (Bausch and Lomb Inc. Stereo Zoom 7), at 70X magnification, and ocular micrometer was used to examine leucocrit. The leucocrit value was calculated as the height of the 'buffy' layer divided by the height of the total blood column times 100 equals percent leucocrit.

After blood samples were examined, all fish were weighed to the nearest 0.1 g wet weight and fork length was measured to the nearest 0.1 cm. Between tests, the dilution system and the electrode chambers were cleaned with chlorine bleach. Any residual chlorine after the system was flushed was removed by adding sodium thiosulfate solution and further flushing the system overnight with dilution water.

I. Statistical Analyses

Responses were compared by one-way analysis of variance (ANOVA) (Steel and Torrie 1960) to discern significant heterogeneity due to zinc exposure. The percent mortality data from the embryo-larval test was subjected to arcsin transformation before analyses were performed to arrive at an MATC range. This transformation is recommended with binomial data expressed as percentages when they cover a wide range of values in order to stabilize the variance (Steel and Torrie 1960). Blood parameter values as well as respiratory frequencies were transformed to \log_{10} before comparisons were made in order to stabilize the variance. This conversion is recommended (Snedecor and Cochran 1967) with effects which are proportional rather than additive. Since ventilatory responses were not felt to be

independent events through time, two means were calculated for each fish, one before, and one during zinc exposure. I subtracted the \log_{10} of the during-exposure mean frequency from the \log_{10} of the before-exposure mean frequency and performed the ANOVA on the differences. Blood parameter values were tested with ANOVA comparing the \log_{10} of the hematocrit and leucocrit values of control versus treatment groups. In those tests where there was a significant treatment effect (with either ventilatory data or hematological data), a further analysis was done using the multiple comparison test of Williams (1971) to determine which zinc concentrations caused significant effects. This analysis was based on the assumption of monotonically ordered responses such as are found in a dose response experiment. This assumption makes this procedure more powerful than other multiple comparison tests.

IV. RESULTS

A. Embryo-Early Juvenile Test

Percent mortality values in the embryo-larval exposure ranged from 2.4 to 92.6 % in individual aquaria. The three lowest zinc concentrations (86, 148, and 262 $\mu\text{g}/\text{l}$) caused lower mortalities than did the ambient control water or the two highest zinc concentrations (444 and 819 $\mu\text{g}/\text{l}$). Statistical results (ANOVA and Williams multiple comparison tests) indicated an MATC of 444 - 819 $\mu\text{g}/\text{l}$ Zn (Table V). One-way ANOVA demonstrated significant heterogeneity at the 0.01 level. Final weights of survivors were not correlated with zinc concentration in this test.

B. Respiratory Monitoring

Examination of the strip chart records for zinc-exposed fish reveals the individual variability in ventilatory patterns among fish (Figure 7). Five of the ten short-term tests (1, 2, 8, 9, and 10) showed statistically significant effects at the highest zinc concentrations employed (Table VI). The other five tests (3-7) did not yield significant alterations in ventilatory frequency at any of the test concentrations. Combining results of replicate tests 1 - 3 produces a detection range between 117 and 144 $\mu\text{g}/\text{l}$ with a geometric mean point estimate of 130 $\mu\text{g}/\text{l}$ Zn. Combining replicate tests 6 - 8 results in a detection range of either >814 $\mu\text{g}/\text{l}$ or between 419 and 781 $\mu\text{g}/\text{l}$ with a geometric mean point estimate of 573 $\mu\text{g}/\text{l}$ Zn. Combining replicate tests 9 and 10 gives a detection range of between 115 and 179 $\mu\text{g}/\text{l}$ with a geometric mean point estimate of 144 $\mu\text{g}/\text{l}$ Zn. However, the medium zinc level did not produce a significant respiratory response in any of the ten tests. Fish size did not significantly affect breathing rates when linearly regressed against fork lengths or condition factors.

TABLE V. PERCENT MORTALITIES FROM EMBRYO-EARLY JUVENILE TEST

| MEASURED ZINC CONCENTRATIONS ($\mu\text{g}/\text{l}$) | MEAN PERCENT MORTALITY (REPLICATE VALUES) |
|--|--|
| 5 | 9.6 (13.1, 6.2) |
| 86 | 3.4 (4.4, 2.4) |
| 148 | 4.9 (6.4, 3.4) |
| 262 | 8.0 (9.9, 6.2) |
| 444 | 17.8 (20.0, 15.5) |
| *819 | 85.6 (92.6, 78.7) |

*Significantly heterogenous at the 0.01 level by Williams multiple range test.

Figure 7. Examples of ventilation patterns from four test fish showing varied respiratory rates. Peak height is determined by ventilatory amplitude, recorder sensitivity setting, and position of fish in relation to electrodes.

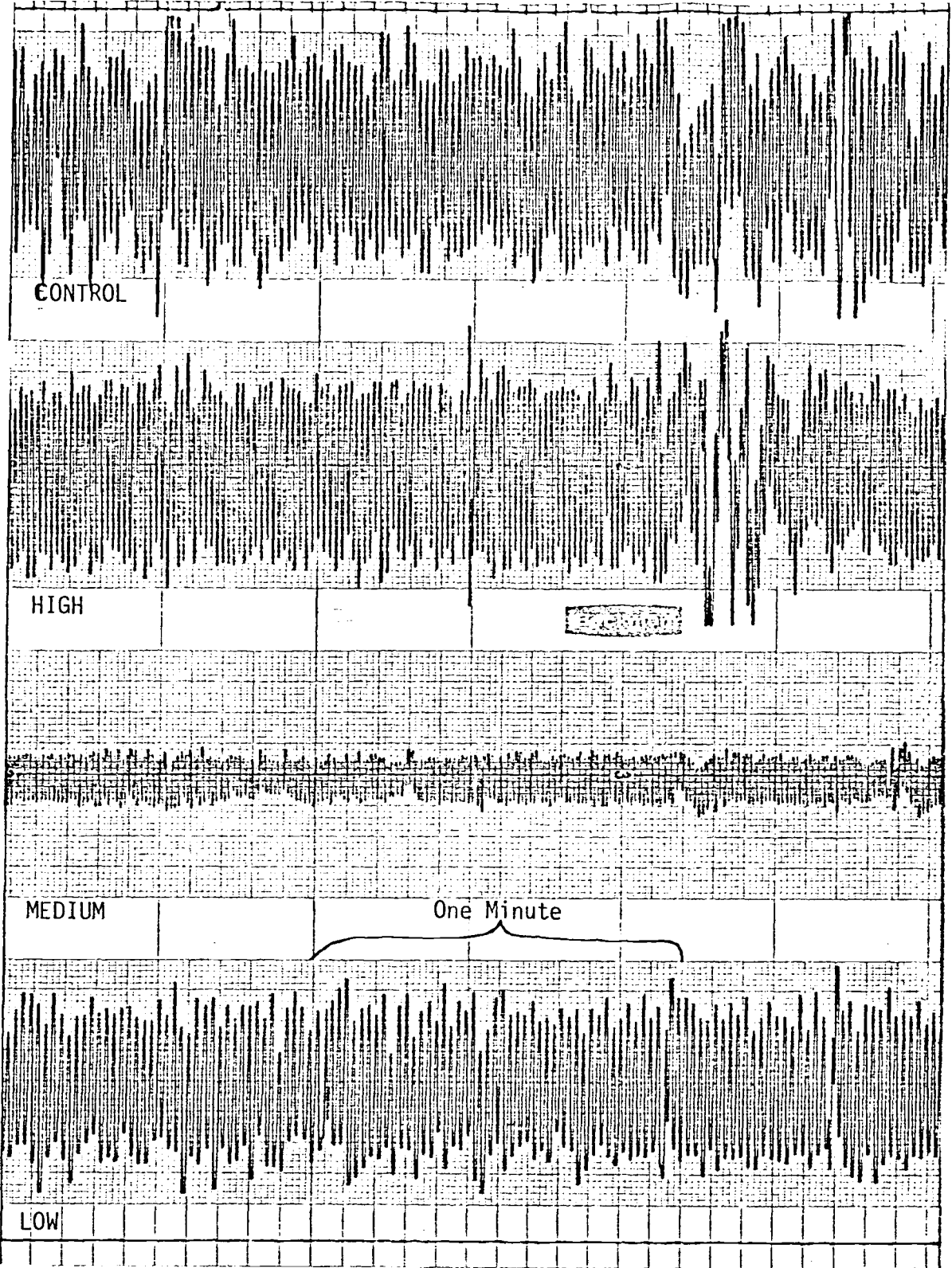


Figure 7.

TABLE VI. RESULTS OF VENTILATORY RATE CHANGES, ESTIMATED DETECTION RANGES, AND MEAN CONTROL PERIOD RATES

| TEST | ANOVA F VALUES (SIGNIFICANCE LEVEL) | WILLIAMS TEST t VALUES ^a (SIGNIFICANCE) | DETECTION RANGE ($\mu\text{g}/\text{l Zn}$) | MEAN CONTROL PERIOD VENTILATORY RATES (+ SD) (min^{-1}) |
|------|---|--|---|---|
| 1 | *3.041 (0.10) | * $t_3 = 2.843$ (0.01) $t_2 = 1.150$ | 94 - 176 | 54 (+ 7) |
| 2 | *6.048 (0.01) | * $t_3 = 3.439$ $t_2 = 0.034$ | 73 - 144 | 62 (+ 10) |
| 3 | 0.826 | b | >117 | 65 (+ 12) |
| 4 | 0.521 | b | >147 | 52 (+ 10) |
| 5 | 0.897 | b | >141 | 62 (+ 6) |
| 6 | 0.981 | b | >814 | 62 (+ 10) |
| 7 | 0.520 | b | >814 | 73 (+ 9) |
| 8 | *4.147 (0.05) | * $t_3 = 1.971$ (0.05) $t_2 = 0.038$ | 419 - 781 | 77 (+ 11) |
| 9 | *3.797 (0.05) | * $t_3 = 2.679$ (0.025) $t_2 = 1.761$ | 115 - 200 | 57 (+ 11) |
| 10 | *3.142 (0.10) | * $t_3 = 2.948$ (0.01) $t_2 = 1.731$ | 91 - 179 | 53 (+ 5) |

^a t_3 refers to the comparison between control frequencies and the highest Zn concentration frequencies; t_2 refers to the comparison between the control frequencies and the medium Zn concentration frequencies.

^b No Williams tests were done when ANOVA demonstrated no significant heterogeneity

* Significantly heterogenous

C. Blood Parameters

In only two of ten tests did zinc exposure produce significant changes in either hematocrit or leucocrit (Table VII). Test 2 showed changes in leucocrit values at all concentrations tested (34, 73, and 144 $\mu\text{g/l}$ Zn). Test 10 indicated changes in both hematocrit and leucocrit values at all zinc concentrations (42, 91, and 179 $\mu\text{g/l}$). All observed blood parameter changes were elevations with respect to control fish values. No combined detection ranges could be determined by adding replicate test results due to the large variability in results among individual tests.

D. Response Levels

All test concentrations were at or below the MATC as determined by the embryo-early juvenile test (444 - 819 $\mu\text{g/l}$ Zn). Using geometric means of the upper and lower limits of the predicted MATC ranges, tests 1 - 3 (Alsea R. stock; 12 C; 25 mg/l hardness) showed detection levels at approximately 22 % of the MATC. Tests 9 and 10 (Santiam R. stock; 12 C; 25 mg/l hardness) show a detection limit at about 24 % of the MATC. Such comparisons are not possible with tests 4 - 8 because of the lack of embryo-larval tests at 7 or 17 C or at 125 mg/l total hardness.

TABLE VII. RESULTS OF BLOOD PARAMETER CHANGES AND DETECTION RANGES

| TEST | ANOVA F VALUES (SIGNIFICANCE LEVEL) | | WILLIAMS TEST t VALUES (SIGNIFICANCE LEVEL) | | DETECTION RANGE ($\mu\text{g/l Zn}$) |
|------|---|-------------------|---|---|---|
| | HEMATOCRIT | LEUCOCRIT | HEMATOCRIT | LEUCOCRIT | |
| 1 | 0.660 | a | b | b | >176 |
| 2 | 0.565 | *5.598 (0.10) | b | * $t_{2c} = 3.216$ (0.025) * $t_1 = 2.664$ (0.025) | 0 - 34 |
| 3 | 0.541 | 1.932 | b | b | >117 |
| 4 | 1.525 | 0.191 | b | b | >147 |
| 5 | 0.066 | 0.683 | b | b | >141 |
| 6 | 1.770 | 0.142 | b | b | >814 |
| 7 | 1.755 | *5.135 (0.025) | | $t_3 = 0.485$ | >814 |
| 8 | 1.489 | 0.218 | b | b | >781 |
| 9 | 1.185 | 1.231 | b | b | >200 |
| 10 | *14.579 (0.005) | *5.796 (0.05) | * $t_3 = 6.132$ (0.01) * $t_2 = 5.126$ (0.01) * $t_1 = 3.365$ (0.01) | * $t_3 = 2.198$ (0.05) * $t_2 = 2.198$ (0.05) * $t_1 = 2.005$ (0.05) | 0 - 43 |

^aNo leucocrit was done in this test.

^bNo Williams test was done when ANOVA indicated no significant heterogeneity

^cLeucocrit values were not obtained for the fish in the highest zinc concentration in this test

* Significantly heterogenous

IV. DISCUSSION

The MATC concept, originally described by Mount and Stephan (1967), is the most useful method for determining survival potential of organisms exposed to water-borne chemicals. The ecological significance of this method lies in the exposure of fish for the entire life-cycle. However, as Macek and Sleight (1977) and McKim (1977) point out, embryo-early juvenile exposures yield nearly identical results in much less time. Data from these tests cannot technically be called MATC's because they are not based on life-cycle exposures although I have used the term MATC in this research. With salmonid fishes, embryo-early juvenile exposures require several months and more rapid test methods to enable accurate predication of long-term chronic effects are needed. Mount (1977) concluded that no research need is greater than the development of rapid and accurate screening methods to estimate toxicity.

The MATC from the embryo-larval exposure in this research (444-819 $\mu\text{g/l}$ Zn) agrees well with that of Holcombe et al (1979) who exposed brook trout to zinc for three generations and reported an MATC of 534-1360 $\mu\text{g/l}$. Benoit and Holcombe (1978) found an MATC of 78-145 $\mu\text{g/l}$ Zn using fathead minnows, Spehar (1976) showed a range of 26-51 $\mu\text{g/l}$ with flagfish (Jordanella floridae), and Sinley et al (1974) reported an MATC of 140-260 $\mu\text{g/l}$ with rainbow trout. All of the above results were obtained from tests in soft water. The latter investigators found an MATC of 36-71 $\mu\text{g/l}$ when fish were not exposed to zinc as eggs. Spehar (1976) and Sinley et al (1974) observed differences in MATC of nearly three and four times between fish exposed as embryos and those exposed first as larvae. This acclimation may be caused by activation of enzymes which either eliminate zinc or control its tissue concentration (Giesy and Weiner 1977). Acclimation to zinc of chronically exposed fish during a relatively resistant embryonic stage causes this phenomenon and probably affected the MATC estimate in this research. Chapman (1978b) reported a 96-hr LC50 for steelhead parr and smolts of 136 and >651 $\mu\text{g/l}$, respectively, using the same dilution water, hardness,

and temperature as was used in the embryo-larval portion of this study. This acute data and the MATC of 444-819 $\mu\text{g}/\text{l}$, determined in this study, results in an application factor of greater than one and indicates no chronic toxicity in soft water. There is little literature on chronic zinc toxicity with salmonids and no evidence of chronic toxicity at concentrations below acutely lethal levels in soft water. Without chronic zinc effects, minimum acute levels are extremely significant.

Neither leucocrit nor hematocrit readings gave results consistent with expected MATC concentrations (Table VII). In eight of the ten tests, the highest zinc concentration failed to elicit a statistically significant alteration of the blood parameters. In test 2, leucocrit was elevated over control values even at the lowest concentration tested (34 $\mu\text{g}/\text{l}$ Zn). In test 10, both hematocrit and leucocrit were elevated at all test concentrations. Mishra and Srivastava (1979) reported that, among other parameters, hematocrit and white blood cell counts were significantly depressed in a freshwater teleost exposed to sublethal zinc concentrations (100 mg/l) for 90 hours. McLeay and Gordon (1977) observed depression of leucocrit values in rainbow trout exposed to stressful conditions for 96 hours and less. They also reported consistent leucocrit depressions in coho salmon (Oncorhynchus kisutch) and rainbow trout exposed for 24 hours to bleached kraft mill effluent. McKim et al (1970) showed that hematocrit was elevated in fish exposed to copper for 6 days but after 21 days the initial changes had disappeared. McLeay (1975) reported that white blood cell-thrombocyte counts were depressed in coho salmon after 24 hours exposure to sublethal concentrations of zinc at 0.5 toxic units and higher. Red blood cell counts were unaffected at up to 1.2 toxic units. He concluded that blood parameters which are very sensitive to pulpmill effluents are not directly applicable to zinc. It may be that because of the nature of the general adaptation syndrome (Selye 1950), the alarm reaction may have passed after 96 hours. It would be interesting to know what exposure time corresponds to the optimum period for a maximum response to this

toxicant. Ecological significance of altered blood parameters is questionable as far as survival value in nature is concerned. Sprague (1976) stated that studies of blood parameters are more suitable for investigating mode of toxic action, but significance is best determined at higher levels of biological integration.

The strength of the bioelectric signal picked up and amplified by the present system varied from 20 to 400 microvolts. This agrees well with the findings of Spoor et al (1971), Camougis (1960), and Roberts (1964). Steelhead trout generally responded to the presence of zinc by aberrations in respiratory patterns. There was considerable variation in frequency, depth, and pattern of the ventilatory signal among individual fish. In general, ventilatory patterns appeared as regular peaks and valleys on the strip chart record. Although not quantified, the presence of zinc caused disruption of the normal pattern resulting in irregular patterns even in fish which did not appreciably increase their ventilatory frequencies (Figure 8). Fish D was exposed to the lowest zinc concentration in test 1 and did not significantly increase its ventilatory frequency. Ventilatory amplitude cannot be measured in this system because it is dependent upon the distance of the fish from the electrodes although some investigators continue to report ventilatory amplitude in similar systems (van der Schalie 1977). My records indicate that ventilatory rate measurement is superior to the more complex and less easily quantified patterns of fish coughs and agree with the conclusion of Maki (1979).

The use of the Williams multiple comparison test with changes (expressed as \log_{10} of ventilatory frequency) in respiratory rate assumes a dose-response effect. When either low or medium concentration varied from the data trend, the values were averaged before the statistical test was performed (Figure 9).

In tests 1 and 2, statistically significant increases in ventilatory frequency occurred at the highest zinc concentration used in each test (176 and 144 $\mu\text{g}/\text{l}$, respectively). In test 3, the actual

Figure 8. Examples of ventilatory patterns from four test fish showing disruption of regular patterns in the fish exposed to the low zinc concentration (D).

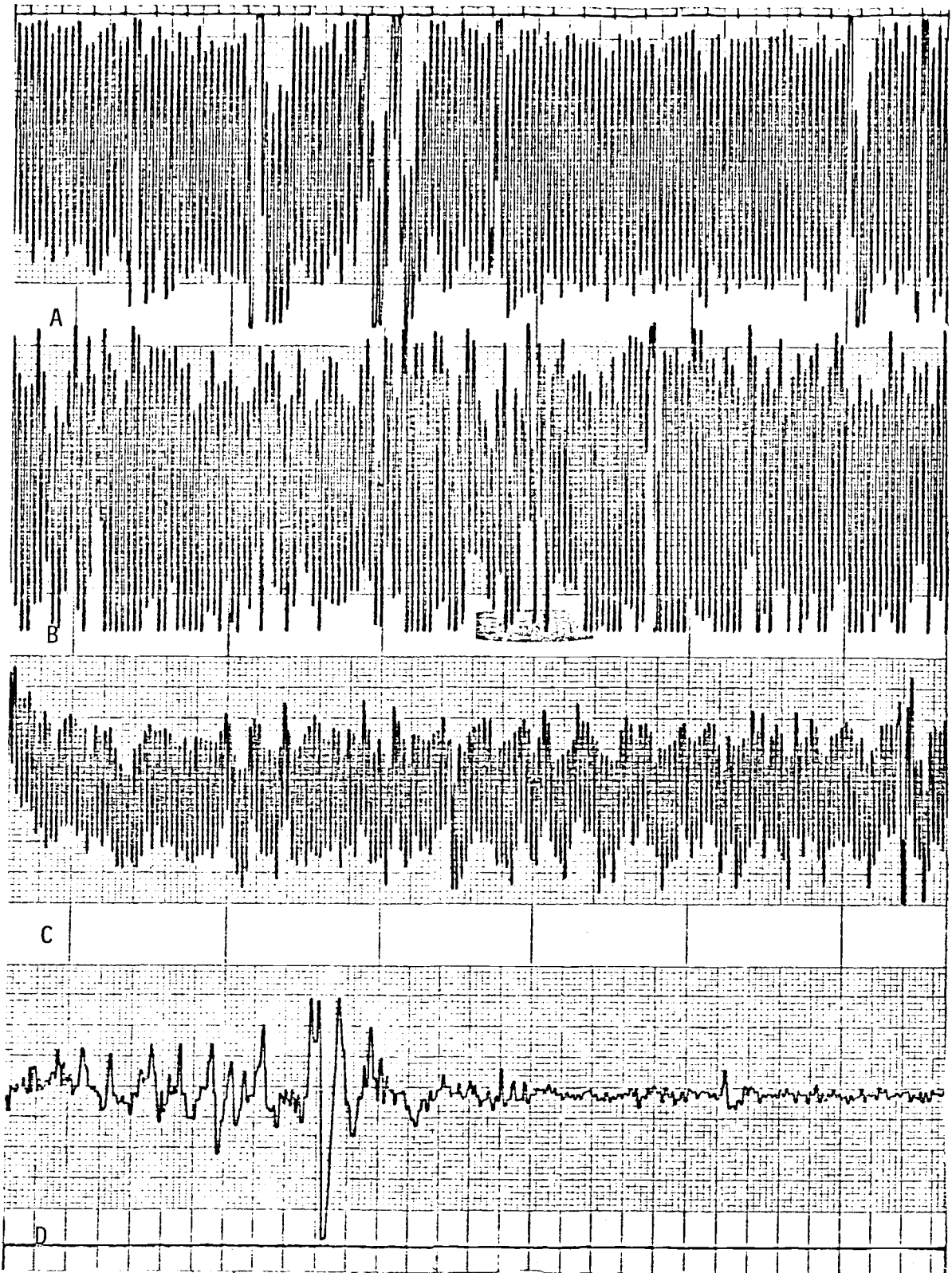


Figure 8.

Figure 9. Plot of zinc-induced changes in mean ventilatory frequency, expressed as \log_{10} .

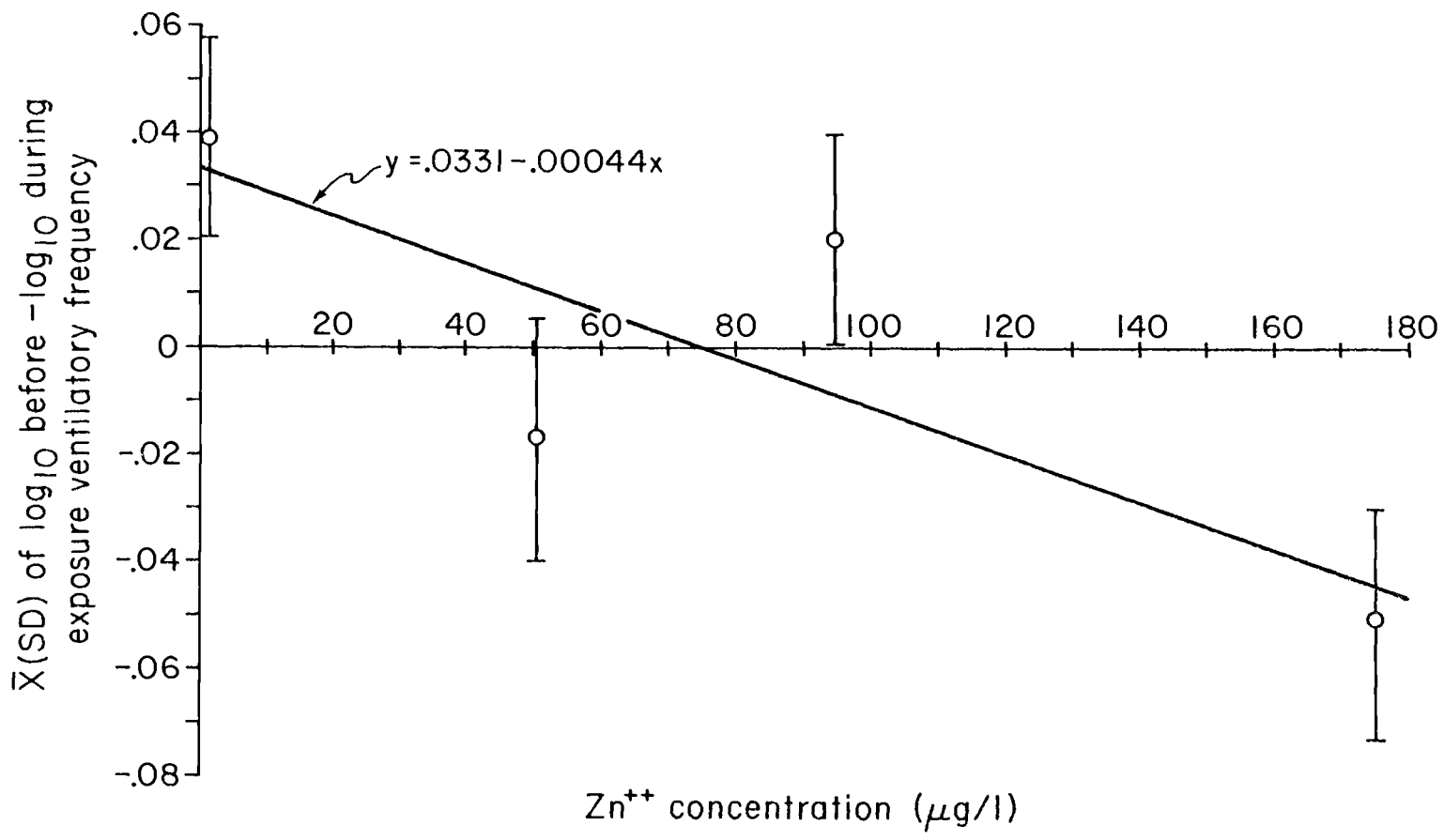


Figure 9.

concentrations dropped significantly below nominals. This was due to a small amount of debris becoming lodged in a toxicant delivery tube. However, the lack of response at 117 $\mu\text{g}/\text{l}$ Zn helps to narrow the predicted MATC range, obtained by combining replicate tests 1, 2 and 3, to 117 to 144 $\mu\text{g}/\text{l}$. This corresponds well to Chapman's (1978b) steelhead parr 96-hr LC50 of 136 $\mu\text{g}/\text{l}$ and is well below my MATC result in the embryo-early juvenile test (444-819 $\mu\text{g}/\text{l}$ Zn). These results indicate that this test system is capable of predicting chronic zinc toxicity to steelhead trout.

In test 4, at 7C., no statistically significant response was observed at the highest zinc concentration (147 $\mu\text{g}/\text{l}$). These concentrations may be below the toxic level and there is a report indicating that temperature alters zinc toxicity. Brown (1968) presented a graph of temperature vs acute toxicity showing that zinc is more toxic at 17 C than at 7 C or 12 C. If this is true it can explain the lack of response in test 4. Cairns and Scheier (1957), however, could show no difference in zinc toxicity to bluegills at either 18 C or 30 C. Lloyd (1960) concluded, from tests with zinc and rainbow trout, that higher temperatures decreased survival times but did not affect threshold concentrations. Stress due to temperature acclimation probably masked the effect due to zinc exposures. I gradually brought chamber temperatures from holding tank temperatures to test temperatures at a rate of less than 3 C per day. Fish were maintained at these temperatures for at least 48 hours before any testing was initiated. Investigators are ambivalent with regard to the necessary time to achieve full temperature acclimation. The Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) recommends at least 2 days maintenance while Doudoroff (1957) states that complete acclimation occurs in less than 3 days, Maki (1979) used 5 days, and Doudoroff (1942) gives a figure of greater than 20 days. Lloyd (1960) indicates that 5 days is adequate, Peterson and Anderson (1969) conclude that 100% acclimation is achieved in 2 weeks or less, and Hodson and Sprague (1975) used greater than 23 days. Cairns and Scheier (1957) used more than 2 weeks, Burton et al (1972) used 1 month, and MacLeod and Pessah (1973) used greater than 2 months for rainbow

trout maintenance.

In tests 6 and 7, fish did not show a significant detectable change in ventilatory frequency at the highest concentration used in the two tests (814 $\mu\text{g}/\text{l}$ Zn). In test 8, a replicate of tests 6 and 7, the fish did respond to the highest concentration (781 $\mu\text{g}/\text{l}$ Zn). These three tests, at 125 mg/l nominal hardness, show that hardness decreases zinc toxicity as has been pointed out elsewhere (Brown 1968; Cairns and Scheier 1957; Holcombe and Andrew 1978; and Lloyd 1960). Although Lloyd (1960) stated that there is no constant ratio between the toxicities of zinc at two specific hardness values, it is known that calcium ions antagonize the action of zinc in fish (Skidmore 1964). Holcombe and Andrew (1978) compared acute zinc toxicity to rainbow trout in hard (176 mg/l) and soft (46 mg/l) water. They noted a 4.5-fold difference in toxicity. Using chronic toxicity values from this study in hard (117 mg/l) and soft (26 mg/l) water, a greater than six-fold difference in toxicity was detected.

It is not known why only one of the three tests detected zinc especially since tests 6 and 7 contained slightly higher concentrations. It would be desirable to continue this avenue of investigation with more tests. It is possible that this system is less able to predict toxic zinc concentrations in hard than in soft water. It is also possible that temperature and/or hardness acclimation time was too short to avoid significant stress which masked the effects of zinc toxicity.

Tests 9 and 10, with Santiam River fish, detected zinc at 115-200 and 91-179 $\mu\text{g}/\text{l}$, respectively. Combining these results gives a range of 115-179 $\mu\text{g}/\text{l}$ Zn which is essentially the same as the range for Alsea River steelhead at the same temperature and hardness. These 2 tests also show the efficient operation of this system insofar as its ability to detect chronically toxic zinc concentrations.

Examination of the mean control period ventilatory rates (Table VI) reveals some effects of hardness, temperature, and strain on respiratory frequency. At 25 mg/l hardness and 12 C, Alsea River fish averaged 60 min^{-1} and Santiam River fish averaged 55 min^{-1} . The Alsea fish were larger at the time of testing and may have suffered

greater confinement stress resulting in a slightly elevated breathing rate. Alsea River fish at 25 mg/l hardness averaged 52 min^{-1} at 7C, 60 min^{-1} at 12 C, and 62 min^{-1} at 17 C. This illustrates the stimulatory effect of temperature on breathing rate. Alsea River steelhead at 12 C averaged 60 min^{-1} at 25 mg/l hardness and 71 min^{-1} at 125 mg/l hardness indicating that the fish increased their breathing rate in response to greater concentrations of calcium ions in the water.

With some toxicants, extermination of the ventilatory increase may occur in time, under continuous exposure, due to an acclimation response by the test fish (Drummond et al 1973; Walden et al 1970; Dandy 1967; and Maki 1979). This tends to negate the utility of the test as an early warning indicator of toxicity in automatic biomonitoring systems. The respiratory data in this study were sub-divided into four 24-hour periods (0-24; 25-48; 49-72; and 73-96) and analysed statistically in the same manner as the 96-hour tests. No evidence for acclimation to zinc effects was seen during the 4-day exposure. The number of 49-72 and 73-96 hour periods containing significant increases in ventilatory rate was as great as the number of 0-24 and 25-48 hour periods showing ventilatory rate increases. Acclimation might occur during a longer exposure period. Perhaps this area warrants further investigation.

The use of fish ventilatory activity has three principal potential uses in water pollution biology. First, because of the Toxic Substances Control Act, many chemical substances must be screened prior to marketing and commercialization. It is essential to have rapid methods to alert aquatic biologists to possible deleterious effects and effect levels so that decisions may be made about further, more extensive testing. Second, P.L. 92-500 requires biomonitoring to be used, where needed, by effluent discharges. Ventilation rate is an easily quantifiable response adaptable to monitoring and an ideal response to study (Cairns et al 1970) if problems such as toxicant acclimation can be resolved. A system such as the one developed in this research could, interfaced with computer technology (Cairns et al 1977) enable industry to regulate effluent treatment and avoid both costly over-treatment as well as potentially disasterous

accident spills. Third, my results indicate that respiratory frequency measurements show a response at or very near chronically toxic concentrations of zinc, and probably other toxicants as well. With proper precautions, the current system can be used to develop water quality criteria.

As Mount and Stephan (1967) and Sprague (1971) point out, any response of an organism to the presence of a toxicant must have ecological significance in nature in order to be relevant in toxicity testing schemes. The ecological relevancy of many tests have yet to be verified by studies of effects in natural aquatic communities due to the complexities and lack of environmental control in such situations. Mount (1977) stated that our overall ability to predict effects resulting from the use of our predictions should be assessed by field monitoring but not for initially measuring acceptable concentrations. Some evidence exists supporting the ecological significance of sublethal tests employing respiratory impairment. Other investigators demonstrated that toxicant concentrations produced respiratory aberrations at or near threshold chronic levels (Maki 1979; Drummond et al 1973; Drummond et al 1974; Walden et al 1970; and Davis 1973). This study also indicates an ability to predict toxicity at concentrations corresponding to chronic values derived from longer life-cycle or critical life-stage exposures. Second, MacLeod and Smith (1966) state that increased coughing frequency (also, presumably, ventilatory frequency) indicates that the organism is undergoing a "loading stress" (Brett 1958) because the expenditure of energy needed for normal maintenance is increased. Ventilatory frequency is an easily quantifiable whole-organism response which integrates stress on all organ systems without behavioral interference created by electrode or cannulae implantations used in other testing systems. This method allows measurement of the collective impact of all environmental factors, which cannot be achieved using even continuous physical and chemical monitoring of individual parameters. Finally, as Warner et al (1966) states, due to the evolution of normal processes, each probably has distinct survival value to organisms in their natural habitats. Therefore, deviation from the norm of any

process is deleterious and one type of change is not necessarily a more valuable indicator than another. Since aquatic organisms are so totally dependent on extraction of oxygen from the aquatic medium, a test method which is concerned with this process must be closely related to the integration of vital life processes.

V. SUMMARY

1. Steelhead trout were exposed to zinc from spawning through 47 days post-hatch and, based on mortality, an MATC estimate of between 444 and 819 $\mu\text{g}/\text{l}$ was calculated.
2. An experimental system was developed to expose steelhead trout to zinc solutions in flow-through chambers for 96 hrs.
3. Bioelectric signals from the fish were monitored in an electronic system consisting of stainless steel electrodes, a multiplex switching device, and a 4-channel strip-chart recorder.
4. Hematocrit and leucocrit values were measured on blood samples taken after the 4-day exposures.
5. Short-term zinc exposures were conducted at three different temperatures, two different total hardness levels, and with two strains of steelhead trout in ten short-term tests.
6. The blood parameters examined were not reliable indicators of chronic zinc toxicity.
7. In five of ten individual tests, ventilatory frequency represented a reliable indicator of chronic zinc toxicity. Coughing frequency was a parameter which required much subjective judgement to quantify and was therefore not analysed.
8. Possible causes for the lack of detection in five of ten short-term tests were: a) sample size may have been too small due to the large individual variability in response among fish; b) temperature and/or hardness acclimation times, especially maintenance time at the test conditions, may have been too short; and c) the test concentrations may have been too low.

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