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Title: SUSCEPTIBILITY OF CHINOOK SALMON (ONCORHYNCHUS TSHAWYTSHA) AND RAINBOW TROUT (SALMO GAIRDNERI) TO INFECTION WITH VIBRIO ANGUILLARUM FOLLOWING SUBLETHAL COPPER EXPOSURE

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Exposure of chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdneri) to sublethal copper concentrations resulted in an increased susceptibility of both species to infection by Vibrio anguillarum. Peak susceptibility after 96 h exposure occurred at 0.09 Toxic Units (TU) of copper for both species. Exposure of rainbow trout to high concentrations of copper (approximately 0.80 TU) resulted in a peak susceptibility after 24 h of exposure followed by a reduction at 96 h exposure with susceptibility returning to control level. Continued exposure resulted in a renewed increase in susceptibility. Exposure to low concentrations of copper (approximately 0.20 TU) also increased susceptibility to infection with a maximum at 48 h exposure and a gradual decline to near control levels after 192 h exposure. It appears that acclimation to the copper-induced stress state can occur

if concentration and duration of copper exposure are not excessive. Copper-exposed and unexposed fish were challenged at a range of bacterial concentrations and LC_{50}'s were determined for the two groups. Exposure of chinook to 0.11 TU of copper for 96 h did not result in a significant change in the number of bacterial cells required to produce an LC_{50} compared to that of non-exposed chinook. However, a significant increase in susceptibility was seen when all the copper-exposed groups were compared to all the non-copper exposed groups. Exposure of rainbow trout to 0.28 TU copper for 48 h resulted in a 48% decrease in the number of bacterial cells required to produce an LC_{50} compared to that required by non-exposed rainbow trout.

Susceptibility of Chinook Salmon (*Oncorhynchus tshawytscha*) and Rainbow Trout (*Salmo gairdneri*) to Infection with *Vibrio anguillarum* Following Sublethal Copper Exposure

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INTRODUCTION

The toxicity of chemical compounds released into the environment has been studied through both acute or short term and chronic or long term bioassays. Whereas the acute bioassay uses survival as its endpoint, the chronic bioassay measures a variety of changes in such things as histopathology, biochemistry, growth, reproductive success, behavior, or numerous others (Sprague, 1971). Often ignored in the chronic studies is the ability of a toxic substance to create a stress state which might manifest itself by a decreased resistance to pathogens.

The ability of pollutants to predispose a fish to disease has been implicated in outbreaks of disease in natural waters either by reactivation of carrier states or by infection with common waterborne pathogens (Wedemeyer, 1970; Snieszko, 1974). Much of the evidence is circumstantial due to the time lag between initial contact with the stressor and the onset of the first mortalities due to the disease organism. The purpose of this work was to investigate the relationship between copper exposure and disease susceptibility of two species of salmonids in a controlled laboratory setting.

Chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Salmo gairdneri) were exposed to copper followed by exposure to a moderately virulent strain of Vibrio anguillarum serotype I. Because this bacterium is not normally found in fresh water (Anderson and Conroy, 1970), the problem of reactivation of latent disease-producing organisms was avoided. The concentrations of copper were usually sublethal and were never greater than the 96 h LC_{50} (concentration killing 50% of test fish). Both chinook salmon and rainbow trout were tested to determine the concentration of copper which produced maximum susceptibility after 96 h exposure. Rainbow trout were also used to determine the length of copper exposure required for an increased susceptibility to infection to occur. Finally, the LC_{50} values of V. anguillarum for copper-exposed and non-exposed rainbow trout and chinook salmon were determined to quantify the effect of the stress.

LITERATURE REVIEW

Toxicity of Copper to Fish

Copper is found in natural waters in various forms. A five-year survey of rivers and lakes in the United States for heavy metals by Kopp and Kroner (1967) revealed that 74% of all samples had measurable copper levels. The mean concentration of soluble copper was 15 µg/ℓ, but the range of values extended to 280 µg/ℓ. Values greater than 20 µg/ℓ were attributed mainly to pollution sources.

Industrial uses of copper include manufacture of electrical products, coins, metal plating, and the production of brass and bronze alloys. The effluents from these factories as well as the corrosive action of water on copper pipe, the leaching of water through mine tailings, and the use of copper oxides and sulfides for pesticides, algicides, and fungicides can lead to increased levels of copper in natural waters (U.S. Environmental Protection Agency, 1976; Kopp and Kroner, 1967). The toxicity of copper to fish varies with the species, size, duration of exposure, and the chemical and physical characteristics of the waters.

Salmonidae appear to be most sensitive to the effects of copper. The 96 h LC_{50} to steelhead trout parr (Salmo gairdneri) and chinook salmon parr (Oncorhynchus

tshawytsha) is 18 and 38 µg/ℓ, respectively (Chapman, 1978). Chronic exposure to low levels of copper has been shown to have deleterious effects on growth, reproduction, survival, and behavior of exposed fish (Mount, 1968; Mount and Stephan, 1969; McKim and Benoit, 1971; McKim et al., 1978).

The method by which copper exerts its lethal effect is not known. Originally, it was assumed that heavy metals coagulated or precipitated the mucus on the gills resulting in interference with oxygen intake (Westfall, 1945). The end result was death by suffocation. Now, however, it appears that copper exerts a much broader effect on the fish and that death may be due to various alterations in the normal body processes.

Baker (1969) held winter flounder (Pseudopleuronectes americanus) in seawater containing various levels of copper. Moribund fish were removed and examined by light and electron microscopy for evidence of tissue damage. Varying degrees of damage were noted, depending on exposure concentrations. High copper caused necrotic areas in the hematopoietic tissue of the kidney and fat accumulation in liver cells. A hemolytic anemia was assumed to be the primary lesion with the liver degeneration a secondary lesion. Destruction of the gill epithelium was also noted and was proportional to the level of

copper exposure.

Degradation of such organs as the liver could result in impaired enzyme systems located within these organs. Jackim et al. (1970) showed that five liver enzymes (acid and alkaline phosphatase, catalase, xanthine oxidase, and ribonuclease) from killfish (Fundulus heteroclitus) were inhibited by copper in an in vitro study. Similarly, all enzymes except alkaline phosphatase were inhibited in vivo.

Lorz and McPherson (1976) demonstrated that Na^+K^+-activated adenosine triphosphatase in gill microsomes of coho salmon smolts (Oncorhynchus kisutch) decreased with increasing copper concentrations. They also observed a decrease in seawater survival with fish exposed to copper, probably due to loss of ability to osmoregulate.

More complete information on the toxic effects of copper may be obtained from the reviews by Doudoroff and Katz (1953), McKee and Wolf (1963), and the USEPA water quality criteria document (1980).

Stress

Hans Selye has worked on the development of a concept of stress since 1936 and has greatly influenced this area of study. His definition that "stress is the non-specific response of the body to any demand made upon it"

has remained one of the simplest (Selye, 1973). Others, in trying to improve on Selye's definition, have proposed quantitative variations such as

> stress is a state produced by any environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that, in either case, the chances of survival are significantly reduced (Brett, 1958).

Similarly, in an attempt to include all living systems and levels of biological organization, Esch et al. (1975) proposed that stress is

> the effect of any force which tends to extend any homeostatic or stabilizing process beyond its normal limit, at any level of biological organization.

When applied at an organismic level, all these definitions address a similar concept that a stressed organism will undergo a physiological response in an attempt to maintain its internal status quo. This response was described by Selye (1950) as the "General Adaptation Syndrome" and is composed of three stages, the "Alarm Reaction" (call to arms of body defenses), the "Stage of Resistance" (adaptation) and lastly, the "Stage of Exhaustion" (loss of adaptation and death).

Mazeaud et al. (1977) in an excellent review of the effects of stress on fish, has divided the responses into two categories, (1) the neuroendocrine or primary effects, and (2) the resulting metabolic and osmotic disturbances or secondary effects. Specifically, the primary

effects were composed of increased levels of plasma catecholamines and corticosteroids. Elevation of these caused changes in tissue water content, heart rate, blood flow, plasma glucose, and lactate, free fatty acids, white blood cell numbers, and an immunosuppressive effect, to name a few.

Pollutant Induced Stress and Disease Interactions

The immunosuppressive action of environmental pollutants has received increased attention. Most of the work, however, deals with effects on mammalian systems. Rabbits fed subtoxic levels of carbamate, organophosphate pesticides, or polychlorinated biphenyls (PCB's) for four weeks demonstrated a dose-dependent decrease in cellular and selected humoral immune responses (Street and Sharma, 1975). Whether this was due to a state of phsyiological stress or some other cause was not clear.

Heavy metals in drinking water have also significantly reduced serum antibody titers to pseudorabies virus in rabbits (Koller, 1973). However, it is not known if this was due to a stress state.

A measurable decrease in the immune response implies an increased susceptibility to infection. Jenson and Rasmussen (1963) demonstrated that mice exposed to high intensity sound stress were more susceptible to

intramuscularly-innoculated vesicular stomatitus virus. This susceptibility appeared independent of adrenal activity. Similarly, a tenfold difference in susceptibility of mice to Salmonella typhimurium organisms was noted after 30 days exposure to sublethal lead contamination (Hemphill et al., 1971).

The effect of environmental stress on disease outbreaks in fish has been reviewed by several authors. Wedemeyer (1970) and Wedemeyer et al. (1976) have discussed the physiological and biochemical manifestations of stress which could affect disease susceptibility in fish. Wedemeyer (1974) also summarized many of the probable stress factors responsible for outbreaks of infectious diseases in intensive culture. Wood (1974) has recorded some of the hatchery conditions which may lead to stress and subsequent disease outbreaks. The literature on marine pollution-associated diseases of fish and shellfish has been extensively covered by Sindermann (1979), and Snieszko (1974) has similarly reviewed the literature concerned with pollution-associated disease of freshwater fish.

The model of Snieszko (1973) that disease outbreaks are due to an interaction of the host, pathogen, and environment is generally accepted. The quantification of such an interaction is difficult in natural systems

because the suspected environmental contaminant which precipitated the epizootic has usually dispersed before measurements can be made.

Indirect evidence of the effect of pollution on disease susceptibility was obtained by Brown et al. (1977) in a survey of a polluted and a non-polluted water system. Fish samples from polluted water had a much higher incidence of non-oncogenic diseases than fish from the non-polluted water.

Pollutant and disease interactions have also been observed within a localized area. An epizootic of Aeromonas hydrophila (A. liquefaciens) in American and threadfin shad (Alosa sapidissima and Dorosoma petenense), was attributed to low dissolved oxygen and altered water quality due to the high biological oxygen demand of cannery discharges (Haley et al., 1967). Industrial effluents such as heavy metals have also been implicated in the induction of localized epizootics. Pippy and Hare (1969) found that an epizootic of A. hydrophila (A. liquefaciens) in Atlantic salmon (Salmo salar) and suckers (Catostomus commersoni) in the Northwest Miramichi River was preceded by a surge of copper and zinc pollution. This pollution, coupled with high temperatures (22.5^{o}C) and low flow, was thought to have precipitated the outbreak. These field studies provide only circumstantial evidence of

pollutant-induced disease. Recently laboratory work has shown effects of pollutant stress on both the immune response and the susceptibility to infection.

Robohm and Nitkowski (1974) found that when cunner (Tautogolabrus adspersus) were exposed to 12 mg/ℓ cadmium for 96 h, they cleared bacteria from the bloodstream faster than non-exposed fish. However, the rate of bacterial killing within the cells of the reticuloendothelial system was significantly reduced. The humoral antibody response was not affected by cadmium because fish exposed to 3 to 24 mg/ℓ cadmium showed no significant difference in antibody production after sheep red-blood-cell injections (Robohm and Nitkowski, 1974).

The exposure of zebrafish (Brachydanio rerio) to 0.1 times the 96 h LC_{50} of zinc for up to four weeks caused a suppression of the humoral antibody response to injected cells of Proteus vulgaris but had no effect on antibody production against infectious pancreatic necrosis (IPN) virus (Sarot and Perlmutter, 1976). Methyl mercury and/or copper, however, when applied at the same level, did suppress the humoral antibody response of the blue gourami (Trichogaster trichopterus) to both Proteus vulgaris and IPN virus injections (Roales and Perlmutter, 1977).

Besides effects on the immune response, increased

disease susceptibility has been noted in laboratory studies of environmental pollutants. Exposure of penaeid shrimp from the northern Gulf of Mexico to polychlorinated biphenyls or mirex increased the prevalence of active infections of a Baculovirus as compared to non-exposed groups (Couch, 1976). European eels (Anguilla anguilla) exposed to 32-60 µg/ℓ copper for 50 days died of vibriosis (Rodsaether et al., 1977). Eels kept in non-contaminated water remained healthy. No bacteria other than Vibrio anguillarum were isolated from the blood of the infected eels. It was assumed that the V. anguillarum had existed in a latent state prior to copper exposure, and the exposure caused a stressed state, triggering the outbreak of vibriosis.

In these cases, the disease agent existed in a latent or carrier state prior to toxicant exposure. Many pathogens present in this state are difficult or impossible to detect. Therefore, determination of the number of infected individuals was not possible in these tests. Recently, controlled laboratory experiments have been conducted to test the theory that pollutants can cause a state of stress manifested by a reduced disease resistance in fish.

Juvenile coho salmon exposed to sublethal levels of chromium (Cr^{++}) in freshwater showed a decreased

resistance to subcutaneously injected V. anguillarum (Sugatt, 1980). The exposure to Cr^{++} increased total mortality and decreased the time to first mortality.

Hetrick et al. (1979) used a water challenge of infectious hematopoietic necrosis (IHN) virus to demonstrate increased susceptibility to virus infection in rainbow trout after copper exposure. Copper concentrations as low as 3.9 µg/ℓ for 1 week resulted in a significant increase in susceptibility. Similarly, Knittel (1981) used a waterborne challenge of Yersinia ruckeri to illustrate that copper exposed steelhead trout were more susceptible to bacterial infection. Exposure to copper concentrations of at least 7 µg/ℓ for 96 h or 10 µg/ℓ for 24 h resulted in significant increases in infection susceptibility.

METHODS AND MATERIALS

Test Fish

Source and Rearing of Experimental Animals

Chinook salmon and rainbow trout were obtained as eyed eggs from the Willamette River Hatchery of the Oregon Department of Fish and Wildlife. Upon arrival at Western Fish Toxicology Station, Corvallis, Oregon, the eggs were disinfected in 1:150 Wescodyne (West Chemical Co. N.Y.), adjusted to pH 7 with $NaHCO_3$, and incubated. After hatching, the fish were reared on a diet of Oregon Moist Pellet (OMP) and were held in circular fiberglass tanks supplied with well water prior to use in experiments.

General Bacterial Procedures

Source and Storage of Bacterial Cultures

Vibrio anguillarum serotype I (LS-174) was chosen for the bacterial infections. The original isolate was obtained in 1974 by Dr. John S. Rohovec (Oregon State University, Department of Microbiology) from fall chinook salmon which died of vibriosis at Lint Slough, Waldport, Oregon. A culture was grown in brain-heart infusion broth (Difco Laboratories Inc., Detroit, MI) and

lyophilized in fetal calf serum. All lyophilized cultures were stored at -10°C until used.

Culture Preparation

For each experiment an ampule of lyophilized cells was innoculated in 5 ml of trypticase soy broth (TSB) and incubated at 18°C for 24 h. This culture was transferred into a larger volume of TSB, incubated at 18°C for 24 h, and used to infect fish.

Experimental Infection Tanks

Infection tanks were 53 cm x 52 cm x 30.5 cm plastic aquaria (Utilitub[14], E. L. Mustee and Sons Inc., Cleveland, OH) fitted with opaque lids to minimize external disturbance of the test fish. Standpipes maintained a constant volume of 46 L, unless otherwise noted. The water was sterilized by UV irradiation, aerated, temperature-controlled by manually operated in-line water heaters, and delivered through PVC pipes from a constant head system to each tank. Flows were 1 L/min for most experiments, and the temperature was held at 16°C. Aeration was applied to each tank to maintain the dissolved oxygen near saturation. Loading densities of fish did not exceed one gram of fish per 3 liters of test water per day as recommended by Sprague (1973). Fluorescent lighting was

provided, and the photoperiod was adjusted weekly to simulate the local photoperiod.

Infection Procedure

A culture of V. anguillarum was grown as described and diluted with sterile TSB to an optical density (OD) of 0.90 at 525 nm in a Bausch and Lomb Spec 88 spectrophotometer. This produced a concentration of approximately 1.0×10^6 cells/ml in the stock culture. Waterflow to the challenge tanks was stopped, and a volume of bacterial culture approximately equal to the LC_{50} dose was added. After 1 h, water flow was restarted to wash out the remaining bacteria. Aeration was maintained continuously during static and flow-through periods.

The exact concentration of bacteria used for each exposure was determined after completion of the challenge by standard plate count methods. The infection was allowed to progress for seven days before the experiment was terminated. Dead fish were removed daily, and were labeled and frozen until necropsy.

Determination of Preliminary Bacterial LC_{50}

The bacterial dose for all succeeding experiments was chosen to be the LC_{50} of V. anguillarum for the fish species being tested and was determined as follows.

Twenty-five chinook salmon (mean weight 6.0 g), fully acclimated to the ambient water temperature (17°C), were added to each of the eight 48 L exposure aquaria by stratified random assignment of groups of five fish each. A culture was prepared, and the fish were infected according to the previously described procedure, except during the flow-through portion of the test the water flow was 1.4 L/min. Duplicate randomized groups of 25 fish were exposed to one of four bacterial concentrations: 0.48, 4.8, 48 and 480 ml of culture (4.3×10^3 to 4.3×10^6 bact/ml).

The LC_{50} determination for rainbow trout (mean weight 20.9 g) was done later when the ambient water temperature was 6°C. Therefore, test fish were moved from the rearing tank to a 946 L acclimation tank where the water temperature was raised to 16°C in daily increments of 1.0-1.5°C, and fish were allowed to acclimate at 16°C for 24 h before transfer to the 16°C experimental aquaria. All fish were fed OMP ad libitum, once daily, but were fasted 24 h prior to transfer. The fish were randomly assigned to the 46 L exposure aquaria as before but were allowed to acclimate for 96 h before the bacterial culture was added to the water. Half of this time they were fed OMP as before; however, they were fasted for 48 h before the start of the experiment.

The bacterial innocula consisted of four duplicate concentrations: 0.46, 4.6, 46 and 460 ml of culture (1.38×10^4 to 1.38×10^7 bact/ml). Only presumptive tests for V. anguillarum infection were done on dead fish. Since these LC_{50} determinations were preliminary to later experimental infections, no separate controls were used.

Necropsy and Isolation of Vibrio anguillarum from Test Fish

Dead fish were externally disinfected in a 10% solution of Wescodyne, and an incision was made just anterior to the dorsal fin to expose the kidney. A sample of kidney tissue was aseptically removed with a sterile innoculating loop and transferred onto two TSA plates. A commercially prepared novobiocin sensitivity disk (BBL, Division of Becton, Dickinson and Co., Cockeysville, MD) was placed on one plate; and a filter paper disk, saturated with the vibriostatic agent 0/129 (2,4, diamino, 6,7 diisopropyl pteridine; Calbiochem, San Diego, CA) and air dried, was placed on the second plate. All innoculated plates were then incubated at 18°C for 48 h.

Inhibition of growth by both novobiocin and 0/129, as well as characteristic colony morphology, were presumptive evidence of V. anguillarum infection. Confirmation was obtained by tests for cytochrome oxidase activity (using

commercially prepared disks, BBL), motility (by the method of Edwards and Ewing, 1972, substituting TSB for the media listed), gram stain reaction, and rapid slide agglutination with rabbit antiserum specific for V. anguillarum (obtained from the Department of Microbiology, Oregon State University). Confirmatory tests were performed on 20% of the presumptive V. anguillarum isolates from each experimental group.

All of the presumptive V. anguillarum isolates subjected to confirmatory tests were identified as V. anguillarum type I; therefore, death was attributed to this bacterium if presumptive evidence was found among the remaining 80% of the experimental animals. Only those dead fish from which V. anguillarum was isolated were considered to have been killed by that bacterium.

Effect of Copper on the Viability of Vibrio anguillarum

In certain experiments, varying levels of dissolved copper were present in the aquaria during bacterial infection. The effect of the dissolved copper on V. anguillarum viability was determined by preparing a range of copper concentrations (0, 4, 10, 20, and 40 µg/ℓ) in duplicate bottles containing 100 ml volumes of sterile well water using a sterile stock solution of $CuCl_2 \cdot 2H_2O$ in distilled water. A 24 h TSB culture of V. anguillarum

was adjusted to OD = 0.90 at 525nm. The copper-spiked water samples were randomized, and 25 ul of diluted V. anguillarum culture was added to each. This approximated the dilution of culture in the aquaria during a bacterial exposure.

Initial plate counts were made in triplicate on samples taken from these mixtures by the spread-plate technique. The samples were serially diluted in sterile phosphate buffered saline (Williams and Chase, 1968), and plate counts were finished within 30 min. of each other to minimize variation.

The mixtures were incubated at 16°C for 1 h to simulate the duration of the infection, and final plate counts were made in the same manner as the initial counts.

A 10 ml water sample was taken from each bottle and placed in an acid-washed, distilled-water-rinsed plastic tube. Each sample was acidified with 0.1% concentrated nitric acid, and the copper concentration was determined on a Perkin-Elmer atomic absorption spectrophotometer equipped with a flameless HGA-2000 heated graphite atomizer.

The remaining water from all bottles was pooled, and total hardness, total alkalinity, and pH were determined according to the methods recommended by American Public Health Association (1971) and the U.S. Environmental Protection Agency (1974).

Copper Exposure

Use of Toxic Units to Express Copper Concentrations

The toxicity of copper is thought to correspond to the concentration of cupric ion (Cu^{++}). Increases in water hardness and alkalinity decrease toxicity by the formation of inorganic complexes with resulting decrease in cupric ion concentration (Chapman and McCrady, 1977; Miller and MacKay, 1980). The water supply used in these experiments was obtained from shallow wells located near the Willamette River and had large variations in alkalinity and hardness during the rainy season dependent upon the stage of the river (Samuelson, 1976).

Chapman and McCrady (1977) prepared a graph showing the effects of increasing hardness in reconstituted water on copper toxicity. Assuming the slope of the line was due to the inorganic complexation of the cupric ion and the intercept was due to the species being studied, the known 96 h LC_{50}'s for both rainbow trout and chinook salmon at a given hardness in the well water were plotted on lines drawn parallel to that of Chapman and McCrady. These lines gave approximations of the 96 h LC_{50} concentrations for both rainbow and chinook at any water hardness.

All experimental copper concentrations are plotted

as toxic units (TU); in the following experiments, 1 TU is equal to the 96-h LC_{50} concentration for the species being tested. The concept of TU is described in detail by Sprague and Ramsey (1965).

Copper Exposure Apparatus

Both chinook salmon and rainbow trout were exposed to copper in aquaria dosed by a continuous flow serial diluter similar to that described by Garton (1980). The following procedures were followed for all copper exposures.

Copper stock solutions were prepared using well water and reagent grade copper chloride ($CuCl_2 \cdot 2H_2O$) and were acidified with 0.1 ml concentrated nitric acid per liter of stock solution. Stock solutions were pumped to a constant head delivery box which supplied a constant volume of copper solution to the diluter.

Total hardness, alkalinity, and pH determinations were normally conducted daily during experiments by the methods recommended by the American Public Health Association (1971) and the U.S. Environmental Protection Agency (1974). Samples for determination of copper concentration were taken daily in 10 ml sample tubes, acidified, and analyzed as described earlier. Mortalities during copper exposure were noted and dead fish removed daily. Water

chemistry data and other pertinent information for each experiment are presented in the Table 1.

Effect of Copper Concentration on Infection Susceptibility

Juvenile chinook salmon were exposed to copper in an aquaria-diluter system isolated from the infection tanks. The diluter delivered 1.5 L/minute to duplicate sets of six aquaria consisting of five concentrations and a control. Aquaria contained 72 L each and were covered by plexiglass and plastic mesh lids. Black plastic shrouds surrounding the aquaria minimized room disturbance. The photoperiod simulated the natural photoperiod using a timer connected to fluorescent and incandescent room lights. The timer was adjusted weekly for seasonal changes in sunrise/sunset.

Twenty chinook were assigned to each aquarium of the diluter by stratified random assignment of groups of five fish. Mean weights for fish in replicate experiments were 9.9 g and 20.2 g. Fish were acclimated to 16^{o}C in the diluter for 96 h. They were fed OMP ad lib for the first 72 h and were fasted for the 24 h prior to the start of copper exposure. Nominal copper concentrations were 0.56, 0.35, 0.20, 0.15, 0.09, and 0.0 TU. Copper exposure was terminated after 96 h. Twenty-five fish at each copper concentration (in lots of 12 and 13

Table 1. Measured Water Quality Parameters and Length-Weight Data for All Copper-Exposed Experiments

Experiment	Rep.	Mean Weight (g) ± S.D.	Mean Fork Length (mm) ± S.D.	Mean Temperature (°C) ± S.D.	Mean Hardness (mg/l as $CaCO_3$) ± S.D.	Mean Alkalinity (mg/l as $CaCO_3$) ± S.D.	Mean pH ± S.D. (Range)
Chinook Varied Concentration Experiment	I	9.9 ± 2.91	91.7 ± 9.12	16.2 ± 0.30	23.5 ± 0.71	25.0 ± 1.41	7.05 ± 0.06 (7.01 - 7.09)
	II	20.2 ± 4.86	117.1 ± 13.5	16.0 ± 0.23	26.3 ± 1.15	26.7 ± 1.15	7.06 ± 0.08 (7.00 - 7.25)
Rainbow Varied Concentration Experiment	I	15.1 ± 5.8	107.0 ± 13.55	16.2 ± 0.36	49.0 ± 3.16	43.0 ± 2.0	6.96 ± 0.09 (6.83 - 7.01)
	II	17.9 ± 3.95	117.8 ± 9.27	16.6 ± 0.45	36.6 ± 5.50	32.2 ± 4.44	6.95 ± 0.14 (6.80 - 7.10)
Rainbow Trout High Copper vs. Time of Exposure	I	20.2 ± 4.30	123.6 ± 10.14	16.6 ± 0.43	36.1 ± 1.46	28.2 ± 2.22	6.98 ± 0.08 (6.91 - 7.05)
Rainbow Trout Low Copper vs. Time of Exposure	I	25.6 ± 5.22	129.7 ± 8.45	17.1 ± 1.71	29.1 ± 0.93	27.4 ± 1.51	7.18 ± 0.13 (6.99 - 7.34)
Chinook LC50 Copper Exposed vs. Non-Exposed	I	8.0 ± 2.06	90.0 ± 7.85	15.5 ± 1.76	23.5 ± 0.55	25.0 ± 1.26	7.04 ± 0.03 (7.00 - 7.05)
	II	8.2 ± 1.58	91.9 ± 4.44	16.2 ± 0.52	23.8 ± 0.75	24.8 ± 0.75	7.01 ± 0.03 (6.96 - 7.03)
Rainbow LC50 Copper Exposed vs. Non-Exposed	I	9.6 ± 2.54	88.4 ± 8.09	15.6 ± 0.31	28.5 ± 0.71	26.5 ± 0.71	6.81 ± 0.05 (6.77 - 6.84)
	II	9.6 ± 2.54	89.2 ± 8.84	15.9 ± 0.24	27.3 ± 2.08	26.5 ± 2.12	6.79 ± 0.02 (6.77 - 6.80)
	III	≈ 10 g	≈ 90 mm	15.8 ± 0.45	23.7 ± 0.58	24.7 ± 2.03	7.08 ± 0.09 (7.02 - 7.18)
	IV	≈ 10 g	≈ 90 mm	16.1 ± 0.43	23.7 ± 2.08	24.8 ± 2.02	7.17 ± 0.01 (7.16 - 7.18)

fish from the duplicate aquaria) were removed from the copper exposure aquaria and carefully transported in 19 L plastic buckets to the randomized 48 L infection tanks. The fish were infected at approximately one LC_{50} dose of V. anguillarum. Necropsy and isolation of V. anguillarum were as previously described.

In addition to the infected fish, a random sample of five fish was taken from each of the five copper concentrations and pooled in a 48 L tank. This served as a stress control group to detect delayed mortality due to copper and received no bacterial innoculum. A well water control receiving no copper or bacterial innoculum was not included in this experiment here but was included in later tests.

Juvenile rainbow trout were exposed to copper in the infection tanks receiving copper dosed water directly from a serial diluter thereby reducing handling stress caused by transfer between separate copper and Vibrio exposure systems. The tanks were also supplied with a freshwater source from a common temperature controlled aeration tank. The diluter and the freshwater system could each supply 1 L/min. to each tank.

Fish were acclimated to the experimental water temperature by the procedure described in the rainbow LC_{50} determination (i.e., 1.0-1.5°C increments daily, fed ad lib,

and fasted 24 h prior to transfer).

Twenty-five temperature acclimated fish (mean weights 15.1 and 17.9 g for two replicates) were randomly assigned in groups of five to each challenge tank and were fed as in previous experiments. An acclimation time of 6-8 days was followed by a 96 h copper exposure. Mean copper concentrations ranged from 0.0 to 0.81 TU and 0.0 to 0.98 TU for replicate experiments.

Following copper exposure, the diluter was shut off, and the fish were exposed to V. anguillarum. Experimental treatments included: 1) copper-stressed groups exposed to V. anguillarum; 2) a non-copper stressed group exposed to V. anguillarum; 3) a copper-stressed group which received 96 h of the highest copper concentration but no bacterial innoculum (stress control); and 4) a non-copper stressed group which received no bacterial innoculum (well water control). Both the rainbow trout and chinook salmon experiments were replicated at later dates since only a single challenge tank was available for each copper treatment.

Effect of Length of Copper Exposure on Susceptibility

A serial diluter was modified to deliver 1 L/min. of a single concentration of copper to each infection tank. The length of copper exposure was regulated by

removing flexible tubes delivering copper dosed water to the tanks and substituting separate freshwater delivery tubes.

Groups of twenty-five rainbow trout were exposed to copper for 0, 1, 2, 4, and 8 days, followed by infection with V. anguillarum. A stress control for delayed copper mortalities (8 day exposure, no innoculum) and a well water control (no copper, no innoculum) were also included in the experiment.

Nominal copper concentrations of 0.80 TU and 0.20 TU were used in the two timed exposure experiments. The longest copper exposure was begun first so that the different treatment groups could all be infected at one time. Mean weights, taken at the end of the experiments, were 20.4 g for the high copper exposure experiment and 25.6 g for the low copper exposure experiment.

Quantification of Copper Stress to *Vibrio anguillarum* by LC_{50}

The LC_{50}'s for juvenile chinook salmon and rainbow trout were determined for fish exposed to a single concentration of copper and for those which were never exposed to copper. Two serial diluters were used, each modified to deliver one copper concentration directly to the infection tanks as in the last experiment. Replicate experiments were done simultaneously.

Juvenile chinook salmon (mean weights 8.0 g and 8.2 g for fish in replicate experiments) were acclimated to test temperature as described before. Fish were randomly distributed to tanks as previously described and acclimated 96 h before copper exposure. Four aquaria served by each diluter received a mean copper concentration of 0.11 TU for 96 h and four received no copper. In addition, a well water control was provided for each system, and a single stress control (0.11 TU copper, no innoculum) was provided for both. The fish were not fed for 48 h prior to copper exposure.

Following 96 h copper exposure, paired bacterial innoculations of tanks were made so that fish in one copper exposed and one non-copper exposed tank of each diluter were exposed to each of four bacterial concentrations (0.46, 4.6, 46, and 460 ml). Standard culture preparation, challenge, and necropsy procedures were followed.

The LC_{50} concentration for rainbow trout was determined in the same manner as for the chinook with the following exceptions: Four replicates wre done rather than two; mean weights for the first two replicates were approximately 9.6 g; mean weights of the latter two replicates were approximately the same; however, exact data are not available; a mean copper concentration of 0.28 TU for 48 h was used to stress the fish, and the lowest bacterial

innoculation was increased from 0.46 ml to 1.0 ml in the latter two replicates.

Statistical Analysis

LC_{50} values for V. anguillarum were calculated using a computer program which linearly regressed percent mortalities (expressed as logits) against the logs of the bacterial concentrations. A similar program was used to regress the percent mortality due to V. anguillarum infection (expressed as logits) against the logs of the copper concentration used as the stressor.

A t-test was used to test for differences in mortality due to V. anguillarum between control and stressed fish in the length-of-exposure experiments. A two way ANOVA using replicates and treatments as classifications and treatment x replicate interaction as an error term was used to analyze the effect of copper on V. anguillarum viability. To determine if significant declines in bacterial numbers occurred through time, the difference between the log initial and log final bacterial concentrations was averaged over all treatments for each replicate. The mean differences were tested with a t-test to determine if they differed significantly from zero.

RESULTS

Preliminary LC_{50} of *Vibrio anguillarum* for Chinook Salmon and Rainbow Trout

Prior to any copper exposure, the LC_{50} value of *V. anguillarum* for chinook salmon and rainbow trout was required because the dosage of bacteria in all following experiments was the concentration of cells/ml which would produce a mortality of approximately 50% in the control groups. The preliminary LC_{50} for chinook salmon was 1.44×10^5 cells/ml at a mean water temperature of $17.1 \pm 0.32^{\circ}C$ (mean ± S.D.). Rainbow trout were less susceptible to this bacterium with an LC_{50} of 2.45×10^5 cells/ml at $16.1 \pm 0.38^{\circ}C$ (mean ± S.D.). These LC_{50} values were found to be variable for both species during the study, possibly due to seasonal or metabolic changes in the host or loss of one or more virulence factors by the pathogen. Therefore, the concentration of bacteria was adjusted for each experiment dependent upon the results of the previous experiment.

Effect of Copper on Viability of *Vibrio anguillarum* during Infection

In many of the experiments, the aquaria contained residual copper solution during the bacterial exposure.

Since a waterborne exposure was used it was important to know if the residual copper in the aquaria had an effect on bacterial viability. This was determined in an experiment simulating aquaria conditions (Table 2). The averages of the log initial minus the log final bacterial concentrations for both replicate experiments were tested (t-Test) to determine if they differed significantly from zero. Both replicates showed a significant decline ($P = 0.05$) in bacterial numbers during the 1 h period in fresh water; however, this decline was independent of copper concentration. A two-way analysis of variance showed no significant effect ($P = 0.05$) of the copper on bacterial viability ($F = 0.61$). The bacteria began to die following addition to freshwater regardless of copper concentration.

Effect of Copper Concentration on Infection Susceptibility

To determine if exposure to sublethal levels of copper prior to infection could influence the disease susceptibility of test fish, chinook salmon were exposed to a range of copper concentrations from 0.0 to 0.56 TU for 96 h. Copper concentrations from 0.08 to 0.20 TU caused the greatest increase in mortality due to vibriosis (Table 3). The highest levels of copper (0.52 and 0.56 TU) did not cause an increase in susceptibility to infection when compared to controls.

TABLE 2. Survival of *Vibrio anguillarum* After One Hour Exposure to Copper in Sterile Well Water.

Copper Concentration (mean ± S.D.) µg/l	Number of V. Anguillarum per ml (x 10^7)				Log Initial Count - Log Final Count	
	0 time Experiment		1 h Experiment		Experiment	
	A	B	A	B	A	B
34.6 ± 1.34	146.33[a]	128.80	144.67	80.53	.005	.204
18.1 ± 1.8	---	108.13	---	89.33		.083
9.4 ± .35	107.20	115.73	68.80	74.20	.193	.193
5.5 ± .21	76.97	123.47	66.03	93.07	.067	.123
<1.3	159.33	121.73	99.23	93.07	.206	.117

[a] Values are averages of 3 standard plate counts.

TABLE 3. Effect of 96 h Exposure to Different Copper Concentrations on the Susceptibility of Chinook Salmon to *Vibrio anguillarum*.

Mean Copper Conc. ±S.D. (µg/l)	Toxic Units	Experiment	Number of Deaths / Number of Fish Tested	No. Positive *V. anguillarum* Isolations	% Mortality due to *V. anguillarum*	LT_{25}[a] (h)
21.1 ± 1.08	0.56	I	10/25	8	32.0	91
12.7 ± 0.05	0.34	I	11/24	10	41.7	88
7.8 ± 0.31	0.20	I	15/25	15	60.0	78
3.1 ± 0.23	0.09	I	10/25	10	40.0	75
0.0[b]	0.00	I	8/25[d]	7	28.0	100
Stress Control[c]		I	0/24[d]	0	0.0	--
20.8 ± 1.04	0.52	II	7/25	7	28.0	92
13.1 ± 2.18	0.33	II	8/24	8	33.3	95
8.1 ± 1.04	0.20	II	8/25	8	32.0	78
6.1 ± 1.34	0.15	II	12/24	12	50.0	75
3.0 ± 0.36	0.08	II	10/25	10	40.0	75
0.0[b]	0.00	II	8/25[d]	8	32.0	100
Stress Control[c]		II	0/25[d]	0	0.0	--

[a] Lethal time to 25% mortality expressed as a percentage of control time.

[b] < 1.0 ug/l.

[c] A five fish sample from each copper concentration except 0.0 µg/l was pooled and not infected. The fish were held to detect copper mortalities occurring after copper exposure was stopped.

[d] Fraction dying following exposure of other treatment groups to *V. anguillarum*.

Vibrio anguillarum was not isolated from fish which received copper only, indicating that the bacterium did not exist in the carrier state in the experimental fish. Delayed copper mortalities (i.e., those appearing after the copper exposure period ended) were not observed in either experimental stress control. However, in Experiment I at 0.56 and 0.34 TU of copper, three fish died following termination of copper exposure; but cultures prepared from them failed to produce bacterial growth. It was assumed that these fish died of copper-induced injuries.

The increased susceptibility to the bacterium is also reflected in the LT_{25} (lethal time to 25% mortality). All chinook exposed to copper showed a decline in the time in which 25% of the test fish died of V. anguillarum infection when compared to their controls. In both replicates, the LT_{25} at the most stressful concentrations (0.08-0.15 TU) was about 25% less than their control LT_{25}.

When rainbow trout were exposed to copper concentrations from 0.0 to 0.98 TU for 96 h (Table 4), the results were similar to experiments with chinook. In experiment I, the maximum susceptibility to V. anguillarum infection was at 0.18 TU whereas at the highest copper concentration (0.80 TU), the susceptibility was slightly less than the controls. The maximum susceptibility in the replicate experiment occurred at 0.31 TU, and again the highest

TABLE 4. Effect of 96 h Exposure to Different Copper Concentrations on the Susceptibility of Rainbow Trout to *Vibrio anguillarum*.

Mean Copper Conc. ±S.D. (µg/l)	Toxic Units	Experiment	Number of Deaths / Number of Fish Tested[a]	No. Positive *V. anguillarum* Isolations	% Mortality due to *V. anguillarum*	LT_{25}[b] (h)
24.0 ± 0.64	0.80	I	9/18	9	50.0	134
18.7 ± 1.08	0.62	I	16/21	15	71.4	93
12.8 ± 0.65	0.43	I	14/21	14	66.7	97
9.9 ± 1.20	0.33	I	18/24	18	75.0	87
5.4 ± 0.56	0.18	I	20/24	20	83.3	82
0.0[c]	0.00	I	14/25	14	56.0	100
Stress Control[d]						
24.2 ± 1.13	0.81	I	3/10[e]	0	0.0	--
Well Water Control[f]						
0.0[c]	0.0	I	0/25[e]	0	0.0	--
25.5 ± 0.86	0.98	II	6/21	4	19.0	94[g]
18.0 ± 0.46	0.69	II	8/24	8	33.3	84
12.9 ± 0.95	0.50	II	5/22	5	22.7	102[g]
8.0 ± 0.44	0.31	II	19/24	19	79.2	62
3.8 ± 0.59	0.15	II	4/25	4	16.0	115[g]
0.0[c]	0.0	II	5/24	5	20.8	100[g]
Stress Control[d]						
25.4 ± 0.94	0.98	II	7/14[e]	0	0.0	--
Well Water Control[f]						
0.0[c]	0.0	II	0/25[e]	0	0.0	--

[a] The number of fish tested is the number alive following copper exposure. All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during the copper exposure.

[b] Lethal time to 25% mortality expressed as a percentage of control time.

[c] < 1.0 µg/l.

[d] Fish exposed to high copper concentration without subsequent infection with *V. anguillarum*. The fish were held to detect copper mortalities occurring after copper exposure was stopped.

[e] Fraction dying following exposure of other treatment groups to *V. anguillarum*.

[f] Fish exposed to well water only without subsequent infection with *V. anguillarum*.

[g] Values extrapolated beyond available data. Less than 25% total mortality occurred during the experiment.

concentration produced little change in susceptibility.

The stress controls in the chinook experiment were a pooled sample of fish taken from all the test concentrations, while the stress controls in the rainbow trout experiments were taken only from the highest concentration. Thirty percent of the stress controls in experiment I and 50% in experiment II apparently died of copper-induced injuries after copper exposure had been stopped. In neither case were bacteria isolated from the dead fish.

In those fish exposed to the bacterium after copper exposure, only at 0.98 TU in experiment II did any delayed copper mortalities occur. All other dead fish showed fulminating infections of V. anguillarum. It is assumed that some of these fish would have died due to copper induced injury alone since they appeared severely distressed at the termination of copper exposure; however, in their debilitated state they succumbed to bacterial infection prior to death induced by copper.

Not all copper exposed rainbow trout exhibited reduced LT_{25} values. Treatments which resulted in increases in susceptibility to infection were accompanied by corresponding decreases in LT_{25} values. Four of the six concentrations in replicate II never reached the 25% mortality level, and the LT_{25} values were obtained by extrapolations. In this replicate, the maximum susceptibility to infection

was accompanied by a 38% decrease in the LT_{25}; however, no concentration related trends were apparent.

Analysis of both the chinook salmon and rainbow trout data by logistic regression showed no significant fit (P=0.10) of a linear model; however, when a quadratic term was introduced in the model, a highly significant fit (P=0.005) of the data was found (Figure 1). This analysis showed peak infection susceptibility occurred at 0.09 TU copper after 96 h exposure. Exposure to higher copper concentrations resulted in a reduction in susceptibility to infection to near that of control.

Effect of Length of Copper Exposure to Infection Susceptibility

The above experiment showed that exposure of chinook salmon and rainbow trout to the highest copper concentration for 96 h resulted in no increase in infection susceptibility. However, when rainbow trout were exposed for varying periods to a concentration of copper which had previously failed to increase susceptibility after 96 h exposure (0.80 TU), it was found that susceptibility to *V. anguillarum* was significantly higher (P=0.05) after 24 h of exposure (Table 5). The susceptibility decreased after 48 h of exposure but remained significantly higher (P=0.10) than the control. Exposure for 96 h was not significantly different (P=0.10) from the control group.

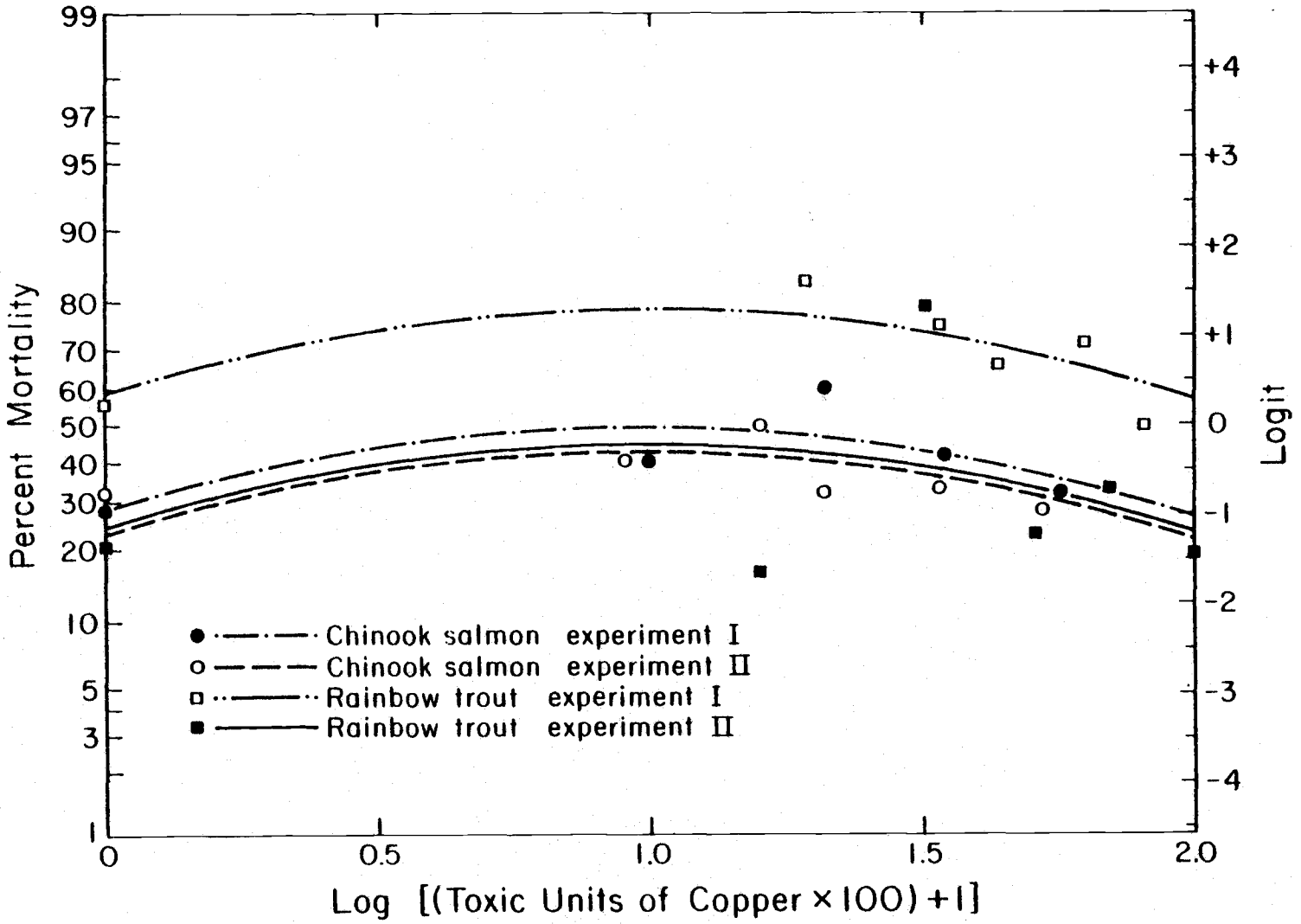

Figure 1. Quadratic regression model showing the effect of 96 h copper exposure on the susceptibility of chinook salmon and rainbow trout to Vibrio anguillarum. Parallel curves fitted for all experiments.

TABLE 5. Effect of Exposure Time at a High Copper Concentration on the Susceptibility of Rainbow Trout to Vibrio anguillarum.

Length of Copper Exposure (h)	Mean Copper Conc. ± S.D. (µg/l)	Toxic Units	Number of Deaths / Number of Fish Tested[a]	No. Positive V. anguillarum Isolations	% Mortality due to V. anguillarum	LT_{25}[b] (h)
192	18.9 ± 2.64	0.76	8/10	8	80.0**	64
96	19.2 ± 0.69	0.77	11/25	11	44.0	102
48	19.9 ± 0.40	0.80	16/24	16	66.7*	94
24	20.7 ± 0.28	0.83	18/24	18	75.0**	76
0	0.0[c]	0.0	10/25	10	40.0	100
Stress Control[d]						
192	18.7 ± 1.88	0.75	0/4[e]	0	0.0	--
Well Water Control[f]						
192	0.0[b]	0.0	0/10[e]	0	0.0	

[a] The number of fish tested is the number alive following copper exposure. All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during the copper exposure.

[b] Lethal time to 25% mortality expressed as a percentage of control time.

[c] < 1.0 µg/l.

[d] Fish exposed to copper for 192 h without subsequent infection. The fish were held to detect copper mortalities occurring after copper exposure was stopped.

[e] Fraction dying following exposure of other treatment groups to V. anguillarum. Only 10 fish were used rather than normal 25 fish in this control.

[f] Fish exposed to well water only without subsequent infection with V. anguillarum.

* Significantly higher than control P = 0.10.

** Significantly higher than control P = 0.05.

Continued exposure to 192 h at this level of copper resulted in a renewed increase in susceptibility to infection. All exposure times, except 96 h showed a decrease in time to 25% mortality indicating a more rapid progression of the infection in the copper exposed groups.

Approximately 60% of the fish died during the 192 h copper exposure, leaving only 10 fish alive at the end of the copper exposure. In the stress control group, which received the same treatment, 84% of test fish were lost prior to completion of the 192 h copper exposure, leaving only four fish to serve as the control for delayed copper mortality. Of these four, no delayed mortalities occurred after copper exposure was terminated. Vibrio anguillarum was isolated from all fish dying after exposure to the bacterium.

A repeat of this experiment at a nominal concentration of 0.20 TU copper resulted in a gradual increase in susceptibility to infection peaking at 48 h exposure (Table 6). At 96 h exposure, an increased susceptibility to infection was still evident. Trends are apparent in this experiment, but they are not statistically significant ($P = 0.10$). The LT_{25} values do not reflect much change in the rate of infection of the copper-exposed groups, except for the 32% decrease seen in the group exposed for 24 h. No delayed copper mortalities were noted in the stress

TABLE 6. Effect of Exposure Time at a Low Copper Concentration on the Susceptibility of Rainbow Trout to *Vibrio anguillarum*.

Length of Copper Exposure (h)	Mean Copper Conc. ± S.D. (µg/l)	Toxic Units	Number of Deaths / Number of Fish Tested[a]	No. Positive *V. anguillarum* Isolations	% Mortality due to *V. anguillarum*	LT_{25}[b] (h)
192	4.28 ± 0.41	0.20	32/45[c]	30	66.7	113
96	4.44 ± 0.19	0.21	19/22	19	86.4	89
48	4.43 ± 0.19	0.21	19/21	19	90.5	98
24	4.20 ± 0.14	0.20	19/22	19	86.4	68
0	0.0[d]	0.0	16/22	16	72.7	100
Stress Control[e]						
192	4.54 ± 0.91	0.22	0/13[f]	0	0.0	--
Well Water Control[g]						
192	0.0[d]	0.0	0/13[f]	0	0.0	--

[a] The number of fish tested is the number alive following copper exposure. All aquaria contained 25 fish prior to copper exposure and deviations from this reflect deaths during the copper exposure.

[b] Lethal time to 25% mortality expressed as a percentage of control time.

[c] Combined total of two aquaria receiving identical treatments.

[d] < 1.0 µg/l.

[e] Fish exposed to copper for 192 h without subsequent infection with *V. anguillarum*. The fish were held to detect copper mortalities occuring after copper exposure was stopped.

[f] Fraction dying following exposure of other treatment groups to *V. anguillarum*. The stress control originally contained 15 fish and the well water control contained 13 fish rather than the standard 25 fish due to a shortage of test fish.

[g] Fish exposed to well water only without subsequent infection with *V. anguillarum*.

control, but two dead fish failed to give V. anguillarum isolations in the group previously exposed for 192 h. These were assumed to be delayed copper mortalities.

Quantification of Copper Stress to Vibrio anguillarum by LC_{50}

When two groups of fish (one exposed to a copper concentration which previously increased susceptibility to infection and one non-copper exposed group) were infected with V. anguillarum at varied doses, the effect of the copper stress or the number of bacterial cells required to produce an LC_{50} was variable. The bacterial LC_{50} concentration for chinook exposed to a mean concentration of 0.11 TU of copper for 96 h was not significantly different ($P = 0.10$) from that of non-copper exposed chinook (Table 7). Exposure to the bacterium either with or without previous exposure to copper resulted in a rapid increase in percent mortality with increasing bacterial concentration until a plateau of infection was reached. Peak mortality from bacterial infection was reached at 1.22×10^5 - 1.47×10^5 cells/ml in fish exposed to copper and 1.47×10^5 - 1.22×10^6 cells/ml in fish not exposed to copper. Further increases in bacterial challenge concentrations over those levels resulted in no additional increase in infection of test fish.

TABLE 7. Effect of Bacterial Dose on Susceptibility of Copper-Exposed and Non-Exposed Chinook Salmon to *Vibrio anguillarum* Infection.

	Number of Deaths / Number of Fish Tested	No. Positive *V. anguillarum* Isolations	% Mortality due to *V. anguillarum*
Bacteria only[a]			
Exp. I			
1.47×10^7	14/25	13	52.0
1.47×10^6	19/25	19	76.0
1.47×10^5	14/24	14	58.3
1.47×10^4	3/25	3	12.0
Exp. II			
1.22×10^7	19/25	17	68.0
1.22×10^6	17/22	17	77.3
1.22×10^5	6/27	6	22.7
1.22×10^4	5/25	5	20.0
Copper + Bacteria[b]			
Exp. I			
1.47×10^7	21/26	21	80.8
1.47×10^6	15/25	15	60.0
1.47×10^5	15/25	15	60.0
1.47×10^4	0/25	0	0.0
Exp. II			
1.22×10^7	24/25	21	84.0
1.22×10^6	13/22	11	50.0
1.22×10^5	21/25	21	84.0
1.22×10^4	0/25	0	0.0
Stress Control[c]	2/25	0	0.0
Well Water Control[d]	0/25	0	0.0

[a] Values are bacterial cells/ml of water in the challenge aquaria.

[b] Mean copper concentration was 4.0 µg/l (0.11 TU) for 96 h.

[c] Fish exposed to 0.11 TU copper without subsequent infection with *V. anguillarum*. These fish were held to detect delayed copper mortalities occurring after termination of copper exposure. A single stress control was used for both replicates.

[d] Fish exposed to well water only without subsequent infection with *V. anguillarum*. A single well water control was used for both replicates.

The data were therefore divided into a series of 2 x 2 contingency tables which were analyzed by the Mantel-Haenszel test to detect any difference between the copper-exposed and non-copper exposed groups (Mantel, 1966). The Mantel-Haenszel test did indicate a significant increase ($P = 0.05$) in the susceptibility of fish exposed to copper and V. anguillarum as compared to those exposed to the bacterium only.

The same experiment was done using rainbow trout exposed to 0.28 TU copper for 48 h before being infected. The LC_{50} for the copper exposed group was 1.41×10^6 cells/ml compared to 2.69×10^6 cells/ml for the non-copper exposed exposed group (Figure 2). The copper exposed group needed approximately one-half the concentration of cell/ml to kill 50% of the test fish as the non-copper exposed group. The difference between the lines was significant at $P = 0.10$.

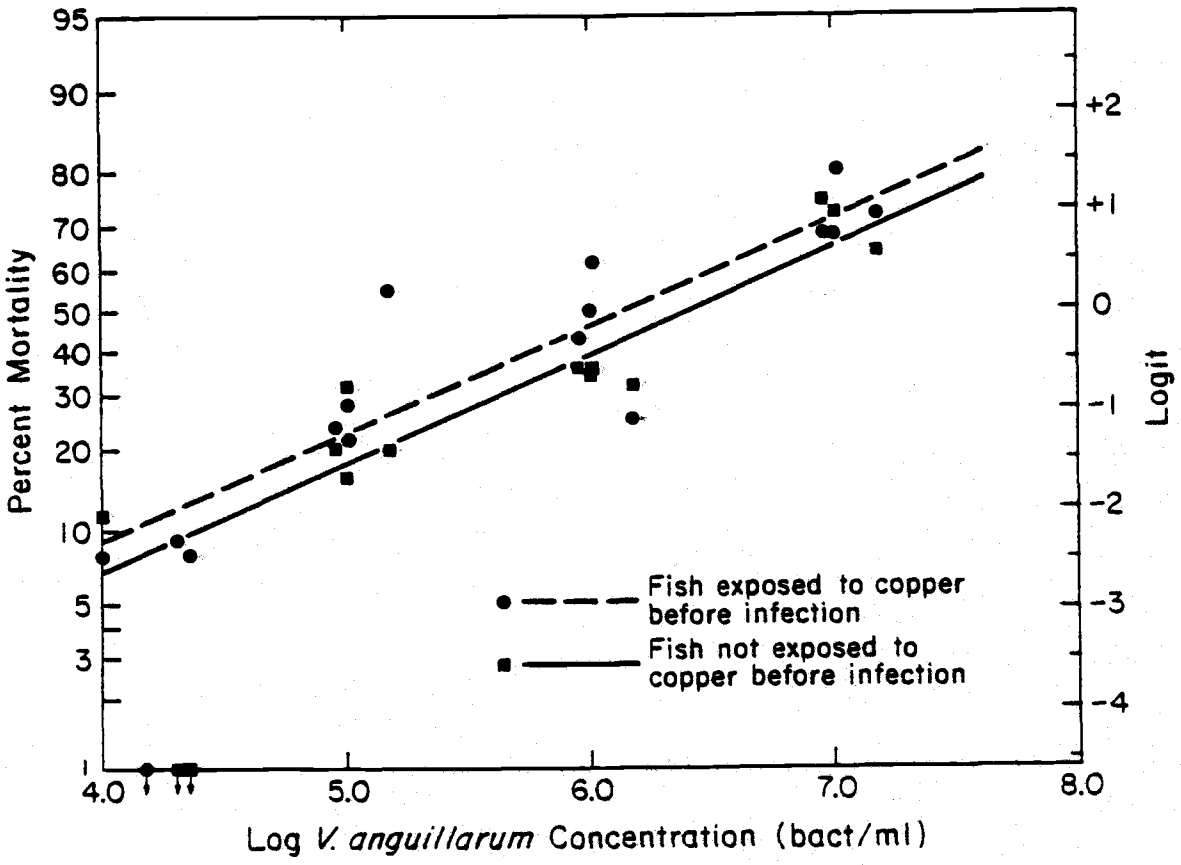

Figure 2. Linear regression showing difference between the LC_{50} to Vibrio anguillarum of rainbow trout exposed to copper and those not exposed to copper. Model shows best fit parallel lines. Mean copper concentrations ± S.D. for exposed fish = 0.28 ± 0.04 TU (5.4 ± 0.61 µg/ℓ) and for unexposed fish = 0.0 TU (<1.0 µg/ℓ). Controls not shown on figure: Stress control for delayed copper mortalilties 0.0% mortality (0/49) at 0.27 TU (5.0 µg/ℓ). Well water control for detecting background toxicity 0.0% mortality (0/49) at 0.0 TU (<1.0 µg/ℓ). Significant difference exists between lines (P = 0.10).

DISCUSSION

The problem of pollutant-induced stress states predisposing fish to infection by pathogens has received increased attention. The study reported here demonstrates the occurrence of increased susceptibility of chinook salmon and rainbow trout to V. anguillarum infection following continuous exposure to dissolved copper. Although this is a marine bacterium which seldom causes problems in freshwater, its consistency in laboratory waterborne infections lends itself to use as a model pathogen for demonstrating pollutant-disease interactions.

Dissolved copper at concentrations found in aquaria during waterborne infections was demonstrated to have no effect on viability of V. anguillarum and, therefore, should not have affected experimental results. Vibrio anguillarum has a definite NaCl requirement and it began to die shortly after exposure to fresh water. Although waterborne exposures were in fresh water, infection occurred rapidly, and no problems were encountered.

Both chinook salmon and rainbow trout were more susceptible to V. anguillarum infection when previously exposed to low concentrations of copper. A linear dose-response curve was not demonstrated, and maximum levels of infection in chinook occurred after 96 h exposure to 0.15 and 0.20 TU of copper. Similarly, rainbow trout

exhibited maximum susceptibility at 0.18 and 0.31 TU of copper in 96 h exposure experiments.

The rainbow trout experiments were more variable than the chinook experiments. This might have been due to changes in the chemical characteristics of the well water (Table 1). The chinook experiments were conducted in the autumn when water hardness and alkalinity values remained constant, whereas the rainbow experiments were performed in early winter during periods of fluctuating water hardness and alkalinity. Water chemistry values were relatively stable throughout the copper exposure itself; however, the effect of fluctuations in water quality in the weeks prior to the experiments is unknown. This could have affected the physiological response of the fish to the toxicant or the bacterium.

The similarity of the four mortality curves prompted the combined analysis of the data for both species by logistic regression. This showed that four parallel curves significantly fit the data and that the susceptibility to infection for both species occurred at 9.0% (0.09 TU) of their respective LC_{50} concentrations of copper. The copper concentrations (as toxic units) causing maximum susceptibility to infection coincided in both species, demonstrating that the percentage of the lethal concentration used to stress fish is more important than the actual concentration.

The recommended safe limit for copper, 0.1 times the 96 h LC_{50} of a sensitive aquatic resident species, as recommended by the U.S. Environmental Protection Agency (1976), is slightly above that concentration which was shown to produce significant increases in susceptibility to infection.

The present criterion is 5.6 µg/ℓ as a 24-h average for total recoverable copper (USEPA, 1980). This, again, is greater than the concentrations which were shown to create an increased state of disease susceptibility in test fish at low water hardness (i.e., chinook salmon parr: LC_{50} at 25 mg/ℓ total hardness as $CaCO_3$ is 38 µg/ℓ. A concentration of 5.6 µg/ℓ is 0.15 TU.) However, since the cupric ion is the most toxic form of dissolved copper (Chapman and McCrady, 1977), and because natural waters normally have a much higher complexing capacity than the well water used in these experiments, a concentration of 5.6 µg/ℓ copper in a river would probably not create a high enough free cupric ion concentration to stress fish and predispose them to disease. Should the complexing capacity of a river be exceeded, the free cupric ion concentration could rise to a level which approaches the concentrations shown in these experiments to produce significant increases in disease susceptibility.

The fish exposed to the higher concentrations of

copper appeared severely stressed prior to exposure to the bacterium but did not show an increased susceptibility to infection. These fish exhibited erratic swimming behavior, gasping at the surface, and a general loss of vitality. It is assumed that had copper exposure been continued, these fish would have died. It was only in these high concentrations of copper that delayed copper mortalities occurred. Following termination of copper exposure, most of these severely-stressed fish recovered within 24 h. It is not evident why these fish, which appeared more seriously stressed than those at the low concentrations, did not show an increase in susceptibility to infection.

Time required for 25% of test fish to die of vibriosis provides a clue as to the mode of increased susceptibility to infection. A decline in LT_{25} values occurred at those concentrations which caused an increased susceptibility to infection, indicating a more rapid progression of the disease in those concentrations. Had the increased susceptibility been solely due to an increase in the number of individuals infected at the challenge, no change would have been seen in the LT_{25} values.

The observation that exposure to high concentrations of copper did not cause increased susceptibility to infection led to the hypothesis that some kind of adaptation

was occurring during the copper exposure period. A phase of resistance following sublethal stress has been postulated by Selye (1950) in his theory of the "General Adaptation Syndrome" (GAS). During this phase, an accommodation presumably occurs which allows the stressed organism to adapt to its stressor, but prolonged contact with a severe stressor may result in an exhaustion of the homeostatic mechanisms, resulting in death of the individual. To determine if acclimation had occurred during the present experiments, rainbow trout were exposed over varying periods of time to a high concentration of copper (approximately 0.80 TU), which previously had not caused increased susceptibility after 96 h exposure, and to a low concentration (approximately 0.20 TU), which previously had caused an increased susceptibility after 96 h exposure. Following exposure to the high concentration, an increased susceptibility noted after 24 h indicated that this concentration was indeed capable of inducing a state of increased susceptibility to infection but that this susceptibility was temporary and was lost after 96 h exposure. The increase again after 192 h probably reflects the exhaustion phase of the GAS. The increase in susceptibility after 192 h exposure to the lower copper concentrations agreed with previous findings. Continued exposure at this low concentration also resulted in an acclimation as reflected

by the decrease in susceptibility at 192 h. It is apparent that both high and low copper concentrations are effective stressors, with effects appearing after only 24 h. The observation in initial experiments that the higher copper concentrations did not result in an increased susceptibility was evidently due to a time and concentration interaction. It appears that both species can adapt to high concentrations of copper provided the duration of exposure is not excessive.

It is possible that it was not an overall acclimation of the fish to copper, but rather a manifestation of a second mode of the immune response which was elicited only after some threshold of toxicity was passed. The use of the term acclimation here is in reference to the reduction in susceptibility and is not meant to infer that this reduction was synonymous with the strict definition of the toxicologist where an acclimation implies an adjustment of the whole animal to one or more environmental factors.

A similar observation of acclimation to copper resulting in decreased susceptibility to infection by fish has also been noted by Hetrick et al. (1979) using IHN virus. In their experiments, exposure of rainbow trout to 10 µg/ℓ copper resulted in significant declines in susceptibility to IHN virus following a peak at 1 day. Although

acclimation appeared to have occurred, susceptibility never returned to the baseline level of the controls as was noted in these experiments. The cause of the acclimation is unknown; possibly it is controlled by hormonal changes, as are many manifestations of the stress response. The concentration of serum cortisol, a principal "stress hormone," has been shown to return to near basal levels within 24 h during exposure of coho to less than 90 µg/ℓ copper (Schreck and Lorz, 1978). They found that at high concentrations of copper (similar in toxicity to those in these tests), the cortisol concentrations rose again after 24 h and remained elevated for the duration of the copper exposure. Therefore, if the decrease in bacterial susceptibility noted in my experiments was due to cortisol levels returning to normal, no acclimation should have been seen when fish were exposed to the high concentrations of copper. It seems unlikely then that the acclimation was due to corticosteroid effects; however, since no cortisol measurements were made in my experiments, the possibility cannot be ruled out.

To determine the effect of copper exposure on the quantity of bacteria needed to kill equal numbers of fish, two simultaneous LC_{50} determinations were done. One LC_{50} was performed on fish immediately after exposure to a previously determined stressful level of copper and one

to a non-copper exposed group. Copper stressed chinook achieved a plateau of infection at a mean concentration of 1.35×10^5 bacteria/ml; increased bacterial levels did not enhance infection. In addition, at approximately 1.35×10^4 bacterial/ml, no infection was noted in the copper-exposed group. Therefore, only a 10-fold increase in bacterial numbers was needed to increase the level of infection in copper-stressed chinook salmon from none to the maximum level attained. In non-copper stressed salmon, the plateau occurred at 1.47×10^5 (Experiment I) and 1.22×10^6 (Experiment II) bacteria/ml. Unlike the copper-stressed group, a small number of fish became infected at the lowest bacterial dose in this group. Therefore, more than a 10-fold (Exp. I) to a 100-fold (Exp. II) increase in bacterial numbers was needed to increase the level of infection from none to the maximum. Because of the plateau effect, the fit of the regression lines was poor and no statistical difference (P=0.10) was shown. The Mantel-Haenszel test was therefore used to determine if a difference actually existed between the copper-exposed and non-exposed group but was hidden by the plateau effect. The copper-exposed group did show a significantly higher susceptibility than the non-exposed group (P=0.05). No plateau effect was seen in experiments with rainbow, and the linear regression of the data showed that the 48%

decrease in bacterial LC_{50} by copper exposed fish was significant (P=0.10). This decrease in the LC_{50} of V. anguillarum by one-half is small compared to that seen by Hetrick et al. (1979) in their work with IHN virus and by Knittel (1981) in his work with Y. ruckeri. It appears that infection by type I V. anguillarum is more dependent upon the virulence of the bacteria than upon the physiological condition of the fish.

How copper acts on the immune system to increase susceptibility to infection is unclear. It is interesting to speculate, however, on some possible mechanisms. It has been shown that exposure of salmonids to sublethal levels of copper causes an increase in the concentration of corticosteroid in the blood (Schreck and Lorz, 1978; Donaldson and Dye, 1975), and that this can result in a lymphopenia in circulating blood presumably due to the lymphocytolytic effect of the steroids (Weinreb, 1958; McLeay, 1973). A decrease in the number of lymphocytes should manifest itself in a decreased humoral response rendering the fish more susceptible to infection. Whereas studies of heavy metal stress have shown some deleterious effect on antibody production in fish (Stevens, 1977; Sugatt, 1980), it is doubtful that the humoral response was a factor in this study. Since all bacterial exposures were terminated at 170 h, no humoral response should have

occurred. This does not, however, rule out the possibility of a lymphocytolytic effect of corticosteroids on the cellular aspects of immunity involving thymus-derived lymphocyte populations.

The increase in susceptibility might also have occurred as a result of interference with the nonspecific immune system. Exposure of cunner (Tautogolabrus adspersus) to toxic or near-toxic levels of cadmium resulted in a significantly slowed destruction rate of bacteria within phagocytic cells of the reticuloendothelial system (Robohm and Nitkowski, 1974). The authors postulated that the lysosomal enzymes were not delivered to the phagosomes due to stabilization of the lysosomal membranes by stress-induced increases to serum corticosteroids. Lysosomal stabilization following stress has also been noted in the alveolar macrophages of stressed rabbits (Lockard et al., 1973).

The direct physical effect of copper on the fish also cannot be overlooked in hypotheses on the mechanism of increased susceptibility. It is known that mucous secretion is increased in fish exposed to heavy metals (Westfall, 1945). Bacterial impingement in the mucous could lengthen contact time needed by a bacterial cell to breach the epidermis of the fish.

It is unknown at present if the increased infection

susceptibility seen in these experiments represents a general effect of pollutants on disease susceptibility or if the increased susceptibility is specific to both copper and V. anguillarum. However, with the volume of circumstantial evidence indicating that pollution predisposes fish to diseases (Snieszko, 1974; Sindermann, 1979), it appears that such effects may have broad implications. With the many thousands of new chemical compounds being produced each year, the probability that some will find their way into streams and rivers is constantly increasing. Since the germ theory itself is based upon the premise that an interaction with normally non-pathogenic bacteria occurs constantly and that it is not until a change in individual resistance occurs that fulminating disease ensues, it seems likely that pollutant induced epizootics could become a more common event in natural water systems. Further, since an acute disease outbreak in severely stressed fish can often occur with little or no external pathology, it appears that the testing of the toxicity of chemicals to fish should include at least a sampling of mortalities for disease organisms. This would ensure that the cause of death in the bioassays was not the reactivation of a latent bacteria carried within the test fish nor the increased susceptibility of the test fish to a common waterborne bacteria.

Further research on the interaction of toxic compounds and disease susceptibility is needed to clarify the method by which the state of decreased resistance occurs. Also, since a highly virulent organism, as was used here, seems to overwhelm the host response, experiments using less virulent organisms and toxicants which produce chronic effects well below acutely lethal levels might be best suited to demonstrate the effects on the host-parasite relationship.

SUMMARY AND CONCLUSIONS

1. Dissolved copper up to 34.6 µg/ℓ has no effect on the viability of V. anguillarum during laboratory waterborne infections.
2. Copper exposure resulted in increased susceptibility of chinook salmon and rainbow trout to V. anguillarum with peak susceptibility after 96 h exposure occurring at 0.09 TU to both species.
3. An increased susceptibility to infection occurred following as little as 24 h exposure to both high copper (0.80 TU) and low copper (0.20 TU) concentrations.
4. Acclimation can occur to the copper, resulting in no increased disease susceptibility if concentration of copper and duration of exposure are not excessive.
5. Increased disease susceptibility was manifested by (1) an increased number of infected individuals and (2) an increased rate of progression of the disease.
6. Exposure of rainbow trout to copper resulted in a decrease by one-half in the number of V. anguillarum cells needed to produce an LC_{50}.
7. Susceptibility to V. anguillarum infection was increased and may have occurred by one or more of the following: (1) through copper-mediated damage to the cellular immune response; (2) through damage

to the non-specific immune response; (3) through a direct physical effect; but not due to damage to the humoral response.

8. Infection by *V. anguillarum* type I appeared to be more dependent on the virulence of the bacterium than upon a copper-induced stress state.

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