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Title: THE EFFECTS OF INTERMITTENT AND CONTINUOUS COPPER EXPOSURE ON THE SUSCEPTIBILITY OF STEELHEAD TROUT (SALMO GAIRDNERI) TO INFECTION BY VIBRIO ANGUILLARUM

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Juvenile steelhead trout (Salmo gairdneri) were intermittently and continuously exposed to copper followed by exposure to waterborne Vibrio anguillarum at 12°C. Intermittent exposures of juvenile steelhead trout from Alsea River Hatchery stock to 1, 2, and 4 pulses of copper (11.8-14.6 µg/l) with a 4-hour pulse duration for parr and a 12-hour pulse duration for smolts did not affect percent mortality to V. anguillarum. When Alsea River steelhead trout were continuously exposed to 14.5 µg/l of copper for time intervals ranging from 12-48 hours there was no effect on percent mortality due to infection. Percent mortality due to V. anguillarum of steelhead parr from Cole Rivers Hatchery stock exposed to copper for 48 hours over a range of concentrations (5.9-33.4 µg/l) or to a single copper concentration (13.4 µg/l or 20.6 µg/l) over a range of times (12-96 hours) was not

consistently different from fish that were not exposed to copper. Finally, exposure of Cole Rivers steelhead parr at 18°C to 13.8 µg/l copper prior to introduction of V. anguillarum resulted in a shorter incubation of the bacterium but had a similar effect on percent mortality. Mean day to death due to V. anguillarum was unaffected by treatment. Most copper concentrations were sublethal but in several treatments mortality due to copper toxicity ranged from 12.2% to 43.3% without affecting susceptibility of the surviving population to V. anguillarum when compared to fish that were not exposed to copper. These results differ from those in similar investigations by other researchers and suggest that stressors do not have a uniform impact on the immune system of fishes. Other factors such as genetics, age, nutrition, temperature and characteristics of the pathogen may be equally important for survival.

The Effects of Intermittent and Continuous Copper Exposure on the Susceptibility of Steelhead Trout (*Salmo gairdneri*) to Infection by *Vibrio anguillarum*

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THE EFFECTS OF INTERMITTENT AND CONTINUOUS COPPER EXPOSURE ON THE SUSCEPTIBILITY OF STEELHEAD TROUT (SALMO GAIRDNERI) TO INFECTION BY VIBRIO ANGUILLARUM

I. INTRODUCTION

Management of toxic substances in the aquatic environment for the protection of fish and invertebrate populations is primarily based on prevention of acute and chronic toxic effects (Environmental Protection Agency, 1976; McKee and Wolf, 1963). Acutely toxic concentrations cause short-term mortality and chronic sublethal toxicity is usually not a direct cause of death but may influence the ability of an organism to reproduce, grow, or behave normally (Warren, 1971). Decreased resistance to infectious disease may be another result of chronic concentrations of a toxicant (Sprague, 1971). Many fish kills attributed to pollution lack data about the specific effects of the chemicals involved upon the fish and how these chemicals may or may not promote invasion of the fish by disease organisms (Stevens, 1977).

An interdependent relationship exists between an aquatic organism, its environment, and potential pathogens. A pathogen may be uniformly present without diesase until a predisposing change in the environment and/or physiological condition of the host confers an advantage to the infectious agent (Snieszko, 1974). The relationship between the environment and diseases in fishes has been reviewed by Snieszko (1974) and Sinderman (1979).

Epizootics due to pathogenic bacteria have been reported to be initiated in fish populations by low dissolved oxygen concentrations (Haley et al., 1967), elevated copper concentrations (Rodsaether et al., 1977), crowding (Wedemeyer and Wood, 1974), sewage and industrial pollution (Mahoney et al., 1973; Shotts et al., 1972), and joint toxicity from copper and zinc along with elevated temperatures (Pippy and Hare, 1969). Brown et al. (1977) have investigated the relationship between environmental quality and diseases in natural populations and observed that the incidence of infectious diseases was 400% greater in a "polluted" Chicago stream than in a similar "non-polluted" stream in Ontario.

Laboratory investigations have linked increased susceptibility to disease with environmental contamination (Rohovec et al., 1980; Walters and Plumb, 1980; Roales and Perlmutter, 1977; Smart, 1976; Butler, 1969; Allison et al., 1964; and Herbert and Merkens, 1961). In a recent investigation, Hetrick et al. (1979) found that rainbow trout (Salmo gairdneri) exposed to sublethal copper concentrations had a percent mortality to waterborne infectious hematopoetic necrosis virus (IHNV) that was twice as great as unexposed controls. Mortality rates due to IHNV generally increased with increasing copper concentrations (below toxic limits) and with increasing potency of the viral dose. The highest percent mortality occurred after one day of copper exposure and subsequent infection and declined in treatments lasting from 3-9 days.

The authors noted that a declining susceptibility with longer copper exposures may be a result of acclimation. Nevertheless, all exposed fish had higher mortality rates than unexposed fish.

Subsequent investigations have also shown an initial increased susceptibility to disease after copper exposure and an acclimation response with extended exposure. Knittel (1980) found that susceptibility of steelhead trout (anadromous rainbow trout) to Yersinia ruckeri increased with time of exposure to sublethal copper concentrations (7-10 µg/l) up to 48 hours, then declined. At 5 µg/l, susceptibility to the pathogen increased during the first 24 hours but was indistinguishable from controls after longer exposures. Baker et al. (1979) reported similar results in salmonids with copper and Vibrio anguillarum and observed an acclimation response after 24 to 48 hours.

Although previous studies have examined the effect of continuous copper exposure on disease susceptibility, fishes under natural conditions may experience fluctuating or intermittent exposures to a toxicant. The toxic effects of intermittent exposure are not well defined but may differ from those of constant exposure depending on the frequency, concentration and duration of the exposure. This study was undertaken to determine whether intermittent exposure of steelhead trout to sublethal copper concentrations affects susceptibility to infection by V. anguillarum.

II. MATERIALS AND METHODS

Fish

Steelhead trout (Salmo gairdneri) hatched in April 1979 at the Western Fish Toxicology Station (U.S. Environmental Protection Agency, Corvallis, Oregon) were used in the first set of experiments. These fish were from Alsea River Hatchery stock, Alsea, Oregon. In the first experiment, the fish still retained their parr markings, but after this they were in the process of smolting. Over the course of experimentation the total length ranged from 13.47 ± 1.72 cm at the beginning to 16.12 ± 2.45 cm in the last experiment.

A second set of experiments used steelhead trout parr from Cole Rivers Hatchery (Trail, Oregon) that were hatched during the 1980 winter. All fish were used prior to the time of smolting. The total length of these fish ranged from 10.74 ± 1.21 cm to 11.81 ± 1.47 cm.

Fish were fed a daily ration of Oregon Moist Pellet until use in an experiment but were not fed during the course of an experiment.

Experimental Design

Experiments were designed to determine the effects of both intermittent and continuous copper exposure on susceptibility of steelhead trout to V. anguillarum.

Experiments involving intermittent exposure to copper consisted of three experimental groups and three control groups. All six treatments were simultaneously replicated. The experimental groups were designed to examine the effect of the number of copper pulses on susceptibility to disease. Therefore, each experimental group was exposed to pulses which were identical in duration, concentration, and frequency but the number of pulses in each group differed. All fish in these treatments were exposed to V. anguillarum immediately after the last copper pulse.

The first control group received only ambient water prior to introduction of V. anguillarum (ambient control). To compare differences between continuous and intermittent exposure, a second control group was continuously exposed to the same copper concentration as the experimental group and subsequently exposed to V. anguillarum (continuous control). The duration of continuous copper exposure varied from 24 to 48 hours between experiments and was chosen to be similar to that which resulted in maximum increases in bacterial susceptibility in previous investigations (Knittel, 1980; Baker et al., 1979). Finally, to assure that mortality was not the result of copper toxicity, one control group received an intermittent copper exposure identical to the experimental treatment with the highest number of pulses but without subsequent exposure to V. anguillarum (copper toxicity control).

Experiments in which fish were continuously subjected to copper before exposure to V. anguillarum were designed to determine the effect of the duration of exposure to copper at a single concentration. These experiments consisted of copper exposure over four time intervals and included ambient and copper toxicity controls. A similar experiment involved exposures to four different copper concentrations for 48 hours with an ambient control and subsequent exposure of all treatments to V. anguillarum. In all cases the treatments were simultaneously replicated.

The dose of V. anguillarum used in all experiments was a previously determined concentration of cells/ml which should produce a mortality of approximately 50% in the ambient control group.

In experiments with Alsea River steelhead, 25 fish were placed in each of twelve aquaria and those experiments employing Cole Rivers steelhead had 40 fish per treatment. Sample size was consistent with loading densities discussed by Sprague (1969). Each fish was cold-branded to identify its treatment and replicate. This method of marking fish does not significantly affect susceptibility to bacterial infection (J. Rohovec, Department of Microbiology, Oregon State University, Corvallis, Oregon, 1980, personal communication). The fish were acclimated to the aquaria for 72-96 hours before experiments began.

Exposure of Fish to Copper

A continuous-flow toxicant diluter developed by Garton (1980) was used for the experiment involving continuous copper exposure over a series of concentrations. This system was modified for all other experiments to deliver an identical concentration to all aquaria receiving copper by eliminating consecutive dilution water flow from the headbox. A further modification provided two aquaria in each replicate with accessory ambient water delivery lines so that they could receive either ambient water or the toxicant. One aquarium in each replicate was provided a separate toxicant delivery pump for continuous exposure during the tests involving intermittent exposure (continuous control).

Using this system all treatments could be simultaneously exposed to V. anguillarum. At the beginning of an experiment copper was delivered to the copper toxicity control and the experimental treatment involving the highest number of pulses or longest continuous copper exposure while all other treatments received ambient water only. During the course of the experiment copper flow was initiated to additional treatments so that copper exposure in all treatments was terminated at the same time. The continuous exposure portion of the system served either as a continuous control during experiments with intermittent exposure or as a fourth interval during continuous copper exposure tests.

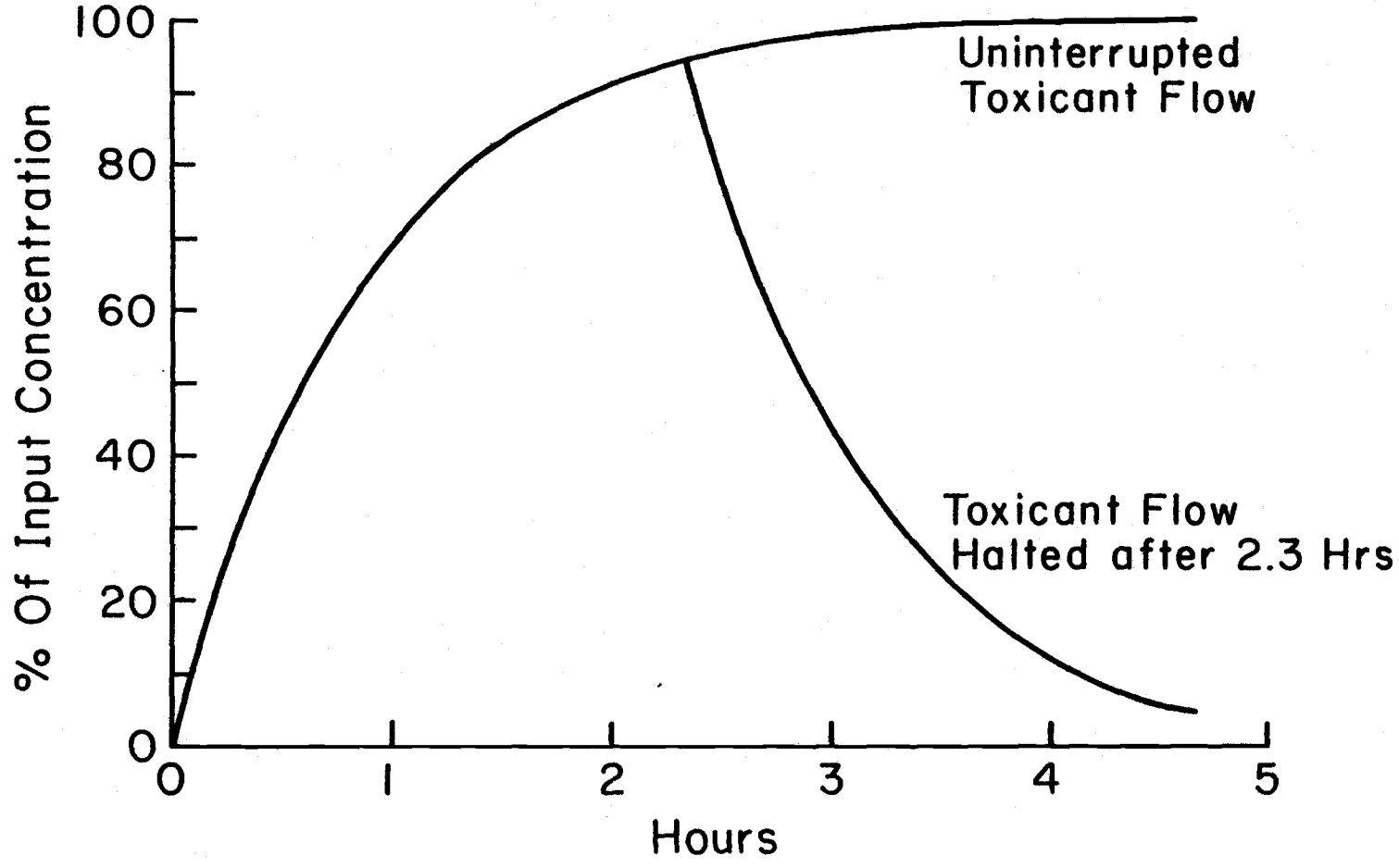
The characteristics of a pulse of copper are a function of the flow rate and the volume of the aquaria which determine the replacement time (Sprague, 1969). A delivery rate of 1.45-1.50 liters/minute into the 67 liter aquaria resulted in a copper concentration that reached 90% of the input concentration in approximately 2 hours (Figure 1). In all experiments, a single pulse of copper was defined as beginning and ending at the ambient concentration.

In experiments with Alsea River steelhead the peak concentration of a copper pulse ranged from 11.8 μg/l to 14.6 μg/l with ambient controls (< 1 μg/l). Preliminary acute toxicity tests indicated that there was significant mortality due to copper toxicity at higher concentra- and similar concentrations to those employed here have caused increased susceptibility to infection in previous investigations (Knittel, 1980; Rohovec et al., 1980; Baker et al., 1979; Hetrick et al., 1979). In an initial experiment employing Cole Rivers steelhead a range of copper concentrations from 0.32 μg/l (ambient) to 33.4 μg/l were tested. In subsequent experiments with these fish copper concentrations ranged from 13.4 μg/l to 20.6 μg/l.

Stock solutions of copper were prepared with reagent grade $CuCl_2 \cdot 2H_2O$. Concentrated nitric acid was added to the stock solution (0.08 ml HNO_3/liter) to prevent precipitation of copper ions. This resulted in a pH change of less than 0.1 pH units in test aquaria.

Actual copper concentrations and pulse characteristics were

Figure 1. Characteristics of a pulse of copper with a delivery rate of 1.50 liters/minute into a 67 liter aquarium.


100
80
60
40
20
0
% Of Input Concentration
0
1
2
3
4
5
Hours
Uninterrupted
Toxicant Flow
Toxicant Flow
Halted after 2.3 Hrs

determined by sampling 10 ml of water taken from each aquarium, acidifying with 0.1 ml of concentrated nitric acid, and analyzing with a Perkin Elmer Model 305B atomic absorption unit equipped with a flameless HGA-2000 heated graphite atomizer. Each aquarium was sampled at least once for each toxicant stock solution during an experiment.

Water temperature was maintained at 12°C during copper exposures for all but the last experiment which employed 18°C. The photoperiod was controlled with a timer and periodically adjusted to the prevailing photoperiod.

Water Chemistry

Daily water samples were analyzed by standard methods for total EDTA hardness, alkalinity, dissolved oxygen concentration (Azide Modification of the Iodometric Method), and pH during copper exposures (American Public Health Association et al., 1965). The chemistry of the well water used in these experiments has been described by Chapman (1978).

Exposure of Fish to *Vibrio anguillarum*

Vibrio anguillarum serotype I (LS-174) from the Department of Microbiology, Oregon State University, was used in all experiments. A stock culture, maintained on Cytophaga medium with 0.5% agar for

up to six months, was the source of bacteria. Maintenance on this low nutrient medium minimized virulence changes due to repeated laboratory manipulations.

Immediately after copper treatment, all fish from each replicate were pooled and moved to a 176 liter stainless steel raceway for exposure to V. anguillarum. In this way all fish within a replicate received the same bacterial dose and were identified by the brand. To determine the effect of transporting the fish between copper and bacterial exposures on disease susceptibility, a fourth control was added in the last two experiments (handling control). Forty fish were acclimated for 96 hours in each of the two raceways used for infection prior to addition of fish from the other treatments and bacterial exposure. The copper toxicity control group was placed in a third raceway but not exposed to V. anguillarum.

Exposure was achieved by pouring an 18-22 hour Tryptic Soy Broth (TSB) (Difco) culture of V. anguillarum, grown at room temperature, into each raceway containing fish from the various treatments. Water in the raceways was held static for 15 minutes after exposure and flow was resumed at a rate of 4 liters/minute. The volume of culture used was determined by time-growth curves for the bacterium in TSB (Gould, 1977) and was designed to achieve an approximate 50% mortality in ambient controls based on preliminary LD_{50} trials. The dose was quantified with plate counts of viable cells (Tryptic Soy Agar)

in the TSB culture (American Public Health Association et al., 1965). The dose of V. anguillarum varied from 2.76×10^5 cells/ml to 1.01×10^6 cells/ml for the experiments at 12°C and was 1.97×10^4 cells/ml in the experiment at 18°C.

Water temperature during exposure to V. anguillarum was 12 ± 1.5°C for the initial experiments and 18 ± 0.1°C for the last experiment. Photoperiod was maintained at prevailing daylength.

Fish dying were collected daily and the kidney tissue was cultured on Tryptic Soy Agar at room temperature for confirmation of the presence of V. anguillarum. Isolates were identified as V. anguillarum by color and morphology of the colony and by subsampling (12-16% of cultures) for rapid-slide agglutination (Bullock, 1971) with rabbit anti-Vibrio anguillarum serotype I (LS-174) antiserum (Department of Microbiology, Oregon State University, Corvallis, Oregon. Only those fish from which the bacterium was isolated were considered to be mortalities resulting from V. anguillarum infection.

Statistics

Data were analyzed as a randomized block design where the blocks were replications. The treatment by replication interaction was used as a measure of experimental error. For experiments with Alsea River steelhead, percentages were analyzed without

transformation, since the range of percent mortality was primarily intermediate between extremes near 0% and 100%. The percent mortality in experiments with fish from the Cole Rivers hatchery was transformed by $y = \sin^{-1} \sqrt{p}$ (Snedecor and Cochran, 1967) for statistical analysis because the range of values in one test was near 100%.

In addition to F-tests, treatments were compared to the ambient control using a t-test. No formal allowance for multiple comparisons was made because of the small number of comparisons involved.

III. RESULTS

Effect of Intermittent Copper Exposure of Short Pulse Duration

Alsea River steelhead parr were subjected to 1, 2, and 4 pulses of copper (12.2 μg/l) with a pulse duration of 4 hours and a frequency of 2 pulses/day to test whether susceptibility to V. anguillarum was increased after short exposures (Table 1). Although susceptibility of fish in experimental treatments of Replicate 2 was as much as 27.5% higher than unexposed fish, fish in the first replicate had a maximum mortality increase of only 6.3%. The continuous control group sustained substantial mortality due to copper toxicity prior to infection but percent mortality due to V. anguillarum (corrected for reduced sample size) was not higher than experimental treatments. There was no significant ($\alpha = 0.05$; F-test, t-test) increase in percent mortality of fish exposed to the intermittent copper pulses or to continuous exposure.

The mean day to death of fish in the continuous control was significantly less than ambient controls ($\alpha = 0.05$; t-test). Bacteria were isolated from the kidney of several fish which died soon after exposure to V. anguillarum and which may have died primarily due to latent copper toxicity. For consistency these were included as deaths due to V. anguillarum in calculation of Mean Day to Death. If these deaths

Table 1. Effects of intermittent copper exposure of short pulse duration[1] on susceptibility of steelhead trout parr (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum[2] at 12°C.
pH = 7.30 Total hardness = 44.5 ± 0.7 mg/l
Dissolved oxygen = 9.35 ± 0.21 mg/l
Peak copper concentration = 12.2 ± 0.6 µg/l
V. anguillarum dose: 1.00 x 10^6 cells/ml

Treatment	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day to Death[3]
0 copper pulses (ambient control)	36.0	12.5	7.33
1 copper pulse	4.0 (0.0)[4]	31.8 (0.0)	7.14
2 copper pulses	39.1 (0.0)	32.0 (0.0)	7.25
4 copper pulses	42.3 (0.0)	40.0 (0.0)	6.73
38 hrs. continuous copper (continuous control)	46.1 (43.3)[5]	37.5 (33.3)[5]	5.42*
4 copper pulses w/o bacteria (copper toxicity control)	(0.0)	(0.0)	

*significant by t-test ($\alpha = 0.05$)

[1]Duration of pulse: 4 hours
Frequency of pulses: 2 pulses/24 hours initiated at 600 and 1600 hours

[2]Nine-day waterborne infection

[3]Mean Day to Death due to V. anguillarum (average of two replicates):

$$\bar{D} = \frac{\sum_{i=1}^{9} iM_i}{\sum_{i=1}^{9} M_i}$$

where: $\bar{D}$ = Mean day to death
i = day of death
M = number of mortalities

[4]represents one mortality

[5]Latent mortality due to copper toxicity occurred for four days after copper exposure. This was before the onset of V. anguillarum infection and no disease organisms were isolated. Percent mortality to V. anguillarum was determined from the population remaining after mortalities due to copper.

are not included in the calculation there is no difference (α = 0.05) between continuous control and the ambient control. In all other treatments, the mean day to death was not significantly (α = 0.05; F-test, t-test) different from the ambient control group.

Effect of Intermittent Copper Exposure of Extended Pulse Duration

Because increased susceptibility to infection using a short pulse of copper could not be demonstrated, several experiments were initiated to determine whether a longer pulse duration would effect susceptibility of steelhead trout to V. anguillarum. Smolting Alsea River steelhead were exposed to 1, 2, and 4 pulses of copper in three experiments at concentrations ranging from 11.8 µg/l to 14.6 µg/l, a pulse duration of 12 hours, and a frequency of 1 pulse/day (Table 2).

In the first experiment, results are reported for one replicate only because fish did not become infected with the bacterium in the second replicate (Table 2, Experiment 1). In this experiment there was a 28% increase in percent mortality of fish after one pulse of copper with decreasing percent mortality after additional pulses. The percent mortality for fish in the continuous control was similar to that of fish in the ambient control.

This pattern was not repeated in the second experiment (Table 2, Experiment 2). In the first replicate the percent mortality of fish in

Table 2. Effects of intermittent copper exposure of extended pulse duration[1] on susceptibility of smolting steelhead trout (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum[2] at 12°C.

Experiment 1: pH = 7.19 ± 0.02 Total hardness = 44.8 ± 0.5 mg/l
Dissolved oxygen = 9.00 ± 0.35 mg/l
Copper = 11.8 ± 0.7 µg/l
V. anguillarum dose: 5.36×10^5 cells/ml

Treatment	% mortality to bacteria[3] (% copper mortality)	Mean Day to Death
0 copper pulses (ambient control)	48.0	5.76
1 copper pulse	72.0 (0.0)	6.33
2 copper pulses	62.5 (0.0)	6.53
4 copper pulses	32.0 (0.0)	6.50
24 hrs. continuous copper (continuous control)	52.0 (0.0)	6.38
4 copper pulses w/o bacteria (copper toxicity control)	(0.0)	

Experiment 2: pH = 7.15 ± 0.03 Total hardness = 27.2 ± 2.2 mg/l
Dissolved oxygen = 9.40 ± 0.14 mg/l
Copper = 14.6 ± 0.8 µg/l
V. anguillarum dose: 9.80×10^5 cells/ml

Treatment	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0 copper pulses (ambient control)	68.2	33.3	7.24
1 copper pulse	38.5 (0.0)	52.0 (0.0)	6.84
2 copper pulses	34.8 (0.0)	____[4]	7.12
4 copper pulses[4]	41.7 (0.0) 52.2 (0.0)	38.4 (0.0) 36.0 (0.0) 44.0 (0.0)	7.15
4 copper pulses w/o bacteria (copper toxicity control)	(0.0)	(0.0)	

Table 2. (Continued)

Experiment 3: pH = 7.20 ± 0.03 Total hardness 29.0 ± 1.4 mg/l
Dissolved oxygen = 9.14 ± 0.29 mg/l
Copper = 14.2 ± 1.0 µg/l
V. anguillarum dose: 1.01×10^6 cells/ml

Treatment	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day To Death
0 copper pulses (ambient control)	60.9	66.7	6.64
1 copper pulse	72.0 (0.0)	52.0 (0.0)	6.58
2 copper pulses	77.3 (0.0)	76.0 (0.0)	6.49
4 copper pulses	60.0 (0.0)	68.0 (0.0)	6.74
24 hr. continuous copper (continuous control)	87.5 (0.0)	[5]	6.28
4 copper pulses w/o bacteria (copper toxicity control)	(0.0)	(0.0)	

[1] Duration of pulse: 12 hours
Frequency of pulses: 1 pulse/24 hours initiated at 800 hours

[2] Nine-day waterborne infection

[3] No infection occurred in Replicate 2

[4] Treatment with 4 copper pulses was inadvertently duplicated within the replicates, eliminating one treatment with 2 pulses and the continuous control.

[5] Treatment was eliminated due to loss of flow through the aquarium and resultant mortality.

the ambient control was 14% higher than that of fish in any experimental treatment. The mortality was 18.7% higher than the ambient control group for fish exposed to one pulse of copper in Replicate 2. With the exception of these treatments fish in all other treatments had similar percent mortalities. There was no continuous control group in this experiment.

Similar results were obtained from the final extended pulse duration experiment (Table 2, Experiment 3). The largest difference in percent mortality between experimental treatments and ambient controls was 16.4% and there was no significant (α = 0.05; F-test, t-test) increased susceptibility to V. anguillarum. Contrary to the results of the first experiment in this series, the percent mortality of fish in the continuous control was 26.6% higher than those in the ambient control.

The results of all three experiments using a 12-hour pulse of copper were analyzed by an F-test and t-test and there was no significant (α = 0.05) effect of treatment on percent mortality of fish to V. anguillarum or on mean day to death.

Effect of Continuous Copper Exposure

Because the results of previous experiments did not show a consistent increased susceptibility of fish to V. anguillarum after copper exposure in intermittent treatments or in the continuous control groups, a series of experiments was designed to determine whether

susceptibility of steelhead trout to V. anguillarum was affected by continuous exposure to copper and whether the length of exposure influenced the response.

Alsea River steelhead smolts were continuously exposed to 14.5 µg/l of copper for 12, 24, 36, and 48 hours prior to introduction of V. anguillarum (Table 3). There was no increased susceptibility of fish to V. anguillarum in any experimental treatment in the first replicate. In Replicate 2, the percent mortality of fish exposed to copper for 36 hours was 12% lower than fish in ambient controls and the mortality in the 48-hour copper exposure was 21.0% higher. But there was no significant effect of treatment on percent mortality (α = 0.05; F-test, t-test). There was also no effect of treatment on mean day to death (α = 0.05; F-test, t-test).

All subsequent experiments were conducted with steelhead parr from the Cole Rivers Fish Hatchery.

The objective of the first experiment using Cole Rivers steelhead was to determine whether the concentration of copper had an effect on susceptibility to V. anguillarum. Fish were continuously exposed for 48 hours to four copper concentrations from 5.9 µg/l to 33.4 µg/l (Table 4). Although the percent mortality in the ambient control group was high the mortality was increased in both replicates for treatments with 10.9 µg/l and 18.3 µg/l. This increase was significant (α = 0.05; t-test) for the exposure to 18.3 µg/l of copper. Additionally, the mean

Table 3. Effects of continuous copper exposure on susceptibility of smolting steelhead trout (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum at 12°C.
pH = 7.44 ± 0.01 Total hardness = 26.0 mg/l
Dissolved oxygen = 9.21 ± 0.16 mg/l
Copper = 14.5 ± 1.1 µg/l
V. anguillarum dose: 8.30×10^5 cells/ml

Hours copper exposure	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0 hours (ambient control)	68.0 (0.0)	52.0 (0.0)	6.98
12 hours	52.0 (0.0)	56.0 (0.0)	7.19
24 hours	60.0 (0.0)	60.0 (0.0)	7.16
36 hours	66.7 (0.0)	40.0 (0.0)	6.92
48 hours	68.0 (0.0)	73.0 (0.0)	6.66
48 hours without bacteria (copper toxicity control)	(0.0)	(0.0)	

[1] Nine-day waterborne infection

Table 4. Effect of 48-hour continuous exposure to four copper concentrations on susceptibility of steelhead trout parr (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum at 12°C.
pH = 7.23 ± 0.02 Total hardness = 25.0 mg/l
Dissolved oxygen = 9.26 ± 0.24 mg/l
V. anguillarum dose: 7.72×10^5 cells/ml (estimate)

Average copper concentration µg/l	% mortality to bacteria (copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0.32 (ambient control)	77.5 (0.0)	84.2 (0.0)	5.59
5.9	91.9 (0.0)	71.8 (0.0)	5.28
10.9	90.0 (0.0)	94.6 (0.0)	5.00*
18.3	97.2 (2.6)*[2]	96.8 (0.0)*	5.38
33.4	77.8 (7.7)	97.1 (7.9)	5.48

*significant by t-test ($\alpha = 0.05$)

[1]Nine-day waterborne infection

[2]Deaths that occurred before the onset of V. anguillarum infection and from which no disease organism was isolated were presumed to be the result of latent copper toxicity.

day to death of fish exposed to 10.9 µg/l was significantly reduced (α = 0.05; t-test). The percent mortality and mean day to death of all other treatments were not significantly different from ambient controls by F-test and t-test (α = 0.05).

Based on the slight increase in susceptibility in the previous experiment, Cole Rivers steelhead parr were continuously exposed to 20.6 µg/l of copper for 12, 24, 48, and 96 hours to determine if this result could be repeated and whether increased susceptibility could be demonstrated with shorter or longer exposures (Table 5, Experiment 1). Although the percent mortality of fish in several experimental groups ranged from 10.2% to 22.5% higher than that of the ambient control groups, the pattern of percent mortality compared to length of copper exposure was not consistent between replicates and there was no significant treatment effect (α = 0.05; F-test, t-test). In addition, the mean day to death was not significantly different in any treatment (α = 0.05; F-test, t-test). Mortality due to direct copper toxicity ranged from 12.2% to 31.4% in the fish exposed to copper for 96 hours but there were no deaths in the copper toxicity control during the period when fish in the other treatments were dying of V. anguillarum.

The results of exposure of Cole Rivers steelhead parr to 13.4 µg/l copper for 12, 24, 48, and 96 hours followed by V. anguillarum exposure are in Table 5 (Experiment 2). There was no significant effect of treatment on percent mortality to V. anguillarum by the

Table 5. Effects of continuous copper exposure on susceptibility of steelhead trout parr (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum at 12°C.

Experiment 1: pH = 7.22 ± 0.02 Total hardness = 21.2 ± 0.5 mg/l
Dissolved oxygen = 9.60 ± 0.17 mg/l
Copper = 20.6 ± 0.8 µg/l
V. anguillarum dose: 1.84×10^5 cells/ml

Hours copper exposure	% mortality to bacteria (copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0 hours (ambient control)	50.0	36.8	6.89
12 hours	72.5 (0.0)	40.0 (0.0)	6.41
24 hours	45.0 (0.0)	30.0 (0.0)	6.74
48 hours	68.4 (2.6)[2]	46.2 (2.5)[2]	6.40
96 hours	45.8 (31.4)[3]	58.3 (12.2)[3]	6.72
96 hours without bacteria (copper toxicity control)	(29.3)[3]	(30.8)[3]	

Experiment 2: pH = 7.25 ± 0.02 Total hardness = 23.3 ± 0.5 mg/l
Dissolved oxygen = 9.48 ± 0.21 mg/l
Copper = 13.4 ± 0.9 µg/l
V. anguillarum dose: 2.76×10^5 cells/ml

Hours copper exposure	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0 hours (ambient control)	40.0	55.0	6.24
12 hours	62.5 (0.0)	75.0 (0.0)	6.62
24 hours	45.0 (0.0)	55.0 (0.0)	6.36
48 hours	67.5 (0.0)	62.5 (0.0)	6.29
96 hours	65.8 (5.0)[3]	59.4 (7.5)[3]	6.51
96 hours without bacteria (copper toxicity control)	(5.3)[3]	(2.5)[3]	
0 hours (handling control)[4]	62.5	40.0	6.72

Table 5. (Continued)

[1]Nine-day waterborne infection

[2]Deaths attributed to copper occurred on the second and third day after copper exposure. This was before the onset of V. anguillarum infection and no disease organisms were isolated.

[3]Deaths attributed to copper occurred between 48 and 96 hours during copper exposure with several deaths due to latent copper toxicity for 24 hours after that. No disease organisms were isolated. There were no deaths in the copper toxicity control during the time of infection in other treatments.

[4]To determine whether transport of fish between copper exposure and bacterial dose affected results, two groups of 40 fish were acclimated to the raceways used for infection for 96 hours and then exposed to V. anguillarum when the other treatments were added to the raceways for infection.

F-test (α = 0.05). Using a t-test the percent mortality of the fish exposed to copper for 12 hours was marginally significant (α = 0.05) when compared to the ambient control group but no other experimental treatments were significant at this level. Mortality resulting from copper toxicity ranged from 5.0% to 7.5% in the fish exposed to copper for 96 hours but no deaths occurred in the copper toxicity control during the infection period. A handling control group was included to determine whether transport of fish between copper exposure and bacterial dosing affected percent mortality; results were not significantly (α= 0.05; F-test, t-test) different from the percent mortality of fish in the ambient controls. Copper exposure did not significantly (α = 0.05) reduce mean day to death of fish in experimental treatments when compared to the ambient control group.

Since the incubation period of V. anguillarum is temperature-dependent, a final experiment was performed at 18°C to determine whether increased susceptibility could be demonstrated with a shorter incubation time. Cole Rivers steelhead parr were exposed to 13.8 µg/l of copper for 12, 24, 48, and 96 hours prior to introduction of V. anguillarum (Table 6). Percent mortality of fish in treatments exposed to copper was not significantly (α = 0.05; F-test, t-test) higher than the ambient control group. However, the percent mortality for the fish in the 96-hour copper exposure and the handling control group was significantly (α = 0.05, t-test) lower than that of the

Table 6. Effects of continuous copper exposure on susceptibility of steelhead trout parr (Salmo gairdneri) to subsequent exposure to Vibrio anguillarum at 18°C.
pH = 7.36 ± 0.03 Total hardness = 22.8 ± 0.5 mg/l
Dissolved oxygen = 8.22 ± 0.18 mg/l
Copper = 13.8 ± 0.6 µg/l
V. anguillarum dose: 1.97×10^4 cells/ml

Hours copper exposure	% mortality to bacteria (% copper mortality) Replicate 1	Replicate 2	Mean Day to Death
0 hours (ambient control)	32.5	42.5	3.94
12 hours	22.5 (0.0)	28.2 (0.0)	3.96
24 hours	32.5 (0.0)	27.5 (0.0)	4.24
48 hours	35.0 (0.0)	32.5 (0.0)	4.14
96 hours	17.9 (2.5)*[2]	23.1 (2.5)*[2]	4.22
96 hours without bacteria (copper toxicity control)	(0.0)[2]	(2.5)[2]	
0 hours (handling control)	20.0*	20.0*	3.88

*significant by t-test ($\alpha = 0.05$)

[1] Six-day waterborne infection

[2] Mortalities attributed to copper occurred during the last day of copper exposure. No disease organisms were isolated. There were no latent mortalities in the copper toxicity control during the time of infection in other treatments.

fish in the ambient control. Mean day to death was unaffected (α = 0.05; F-test, t-test) by treatment.

IV. DISCUSSION

The experiments reported here were designed to test the effect of intermittent and continuous copper exposures on the susceptibility of steelhead trout to infection by V. anguillarum as measured by percent mortality and mean day to death. The final percent mortality within a treatment represented the proportion of fish which were unable to defend against invasion and replication of the pathogen. Mean day to death was a measure of the time necessary for V. anguillarum to overcome the defense mechanisms of the host at a given temperature and reflects the host's overall immune competence. Using a similar statistic, Baker et al. (1979) showed that the median survival time (50%) of V. anguillarum infection in fish exposed to copper was shorter than for fish that were not exposed to copper.

Attempts to demonstrate increased susceptibility to V. anguillarum after intermittent exposure to a sublethal copper concentration using percent mortality and mean day to death were unsuccessful. The percent mortality due to V. anguillarum was marked by a lack of uniformity between treatments and replicates. For example, only one fish died in a single 4-hour treatment in the experiment using a short pulse duration (Table 1) and the 12.5% mortality in one ambient control group of the same experiment was also deviative when compared to other treatments. Additionally, the differences in treatment effects,

when compared to the ambient control, were not reproducible from one experiment to the next. In the initial experiment employing a 12-hour pulse of copper, susceptibility to V. anguillarum appeared to be increased after a single exposure but subsequent experiments indicated that this was due to variation within a treatment and not to effects of copper exposure (Table 2). Data for the mean day to death was much less variable, but no statistically significant ($\alpha = 0.05$) treatment effects were observed by either mean day to death or percent mortality.

The copper concentrations employed in the experiments with intermittent exposure (11.8-14.6 µg/l) were similar to those which were shown in previous investigations to cause an increase in susceptibility to several pathogens after 24-48 hours of continuous exposure (Knittel, 1980; Baker et al., 1979; Hetrick et al., 1979). In contrast to those experiments, the percent mortality of fish in the continuous copper exposed controls reported here (Tables 1 and 2) was not consistently different from that of the ambient control groups. Additionally, the final experiment with Alsea River steelhead smolts failed to show an increased susceptibility to V. anguillarum after continuous exposures of 12, 24, 36, and 48 hours (Table 3) at a copper concentration of 14.5 µg/l.

The lack of uniformity of data between replicates and treatments (Tables 1 and 2) and the failure to demonstrate increased susceptibility

to V. anguillarum in the Alsea River steelhead after continuous copper exposure were not fully explained. The process of smolting may have increased the physiological heterogeneity of the population, thereby increasing the probability of highly deviative values with the small sample size that was used. If this were the case, small treatment effects would be more difficult to resolve. In addition, differences in disease resistance (Winter et al., 1980; Snieszko et al., 1959) have been shown to exist between different hatchery stocks of the same species.

To investigate the importance of these factors, experiments using steelhead parr from the Cole Rivers Hatchery were initiated. With these smaller fish, the number of fish per treatment could be increased from 25 to 40. The objective was to determine whether continuous copper exposure increased the susceptibility to V. anguillarum of fish which were genetically and physiologically different from the Alsea River steelhead smolts.

Knittel (1980) and Baker et al. (1979) have demonstrated that the effects of copper on susceptibility of trout to bacterial infection is both time and concentration dependent. Therefore an experiment was designed to determine the effect of continuous copper exposure for 48 hours to four separate copper concentrations. The effect of concentration on susceptibility to V. anguillarum was unclear (Table 4). Although there was no treatment effect on mean day to

death, total percent mortality was higher in both replicates for fish exposed to 10.9 μg/l and 18.3 μg/l of copper. This result was statistically significant (α = 0.05; t-test) for the exposure to 18.3 μg/l but not for concentrations above and below that level. The mean day to death was significantly lower than that of the ambient control group for the treatment receiving 10.9 μg/l of copper. In addition, the percent mortality of fish in the ambient control groups was greater than 75% which limited the potential of the experiment to demonstrate large increases in susceptibility.

Subsequent experiments designed to investigate the effect of length of exposure to 20.6 μg/l and 13.4 μg/l of copper did not result in a consistent pattern of increased susceptibility to V. anguillarum (Table 5). In these two experiments, only the percent mortality of fish in the 12-hour exposure to 13.4 μg/l of copper was significantly (α = 0.05; t-test) higher than the ambient control groups and significance at this level was marginal. It should be noted that if the treatment effects for 48 hours of exposure to copper in the experiment reported in Table 5 are combined with treatments of similar copper concentration in Table 4 for statistical analysis, significance (α = 0.05) is obtained by a t-test in both cases. This is due primarily to the results of the experiment reported in Table 4 and the use of a smaller error term and is not consistently repeated in both replicates of the subsequent experiments (Table 5). Therefore, the results indicate

that copper exposure did not have a meaningful effect on susceptibility to V. anguillarum in these experiments.

Baker et al. (1979) have conducted similar experiments at 16 °C which demonstrated increased susceptibility to V. anguillarum after copper exposure. All experiments previously discussed here were conducted at 12 °C and in most cases there were no deaths due to infection before the fifth day after exposure to V. anguillarum with mortalities continuing through the ninth day. To determine whether the incubation period was affecting results, a final experiment involved continuous exposure to 13.8 µg/l of copper for 12, 24, 48, and 96 hours at 18 °C (Table 6). Deaths due to infection began on the third day after exposure to V. anguillarum and the epizootic was over by the sixth day. As in previous experiments, there was no increased susceptibility due to copper exposure.

The experiments reported here did not demonstrate an increased susceptibility to V. anguillarum after intermittent or continuous exposure to copper. The reasons for the differences between these results and those reported in similar investigations (Knittel, 1980; Rohovec et al., 1980; Baker et al., 1979; Hetrick et al., 1979) are unknown. The effects of copper concentration, length of copper exposure, and water temperature have been discussed. Other factors that have the potential to influence the results of these experiments are the species and stock of fish, the nutritional status of the fish, the age and

physiological condition of the fish, the characteristics of the pathogen, water chemistry, and stresses imposed by experimental methodology which was influenced by researcher judgement.

Increased susceptibility to several pathogens after exposure to copper has been shown in rainbow trout (Rohovec et al., 1980; Baker et al., 1979; Hetrick et al., 1979), steelhead trout (Knittel, 1980), chinook salmon (Oncorhynchus tshawytsha) (Baker et al., 1979), and coho salmon (Oncorhynchus kisutch) (Rohovec et al., 1980). Knittel's results were based on experiments using steelhead trout from the same Alsea River Hatchery as those used here, but they were juveniles which had not yet smolted. In experiments reported here, the use of a genetically different stock of fish from the Cole Rivers Hatchery which were not in the process of smolting also resulted in a negative effect of copper on susceptibility to V. anguillarum. This suggests that the results in this study were not due to genetic differences in the steelhead trout. All fish used were apparently healthy fish which had been fed a normal daily ration of Oregon Moist Pellet.

There may be differences in the effect of copper exposure on susceptibility of fish depending upon the characteristics of the pathogen. Hetrick et al. (1979) demonstrated that percent mortality of rainbow trout to infectious hematopoetic necrosis virus after copper exposure was approximately twice as great as that of unexposed fish for three copper concentrations and exposure times of 1-9 days. Subsequent

investigations with several bacterial pathogens including Yersinia ruckeri (Knittel, 1980) and V. anguillarum (Baker et al., 1979) demonstrated an effect of copper on susceptibility to these pathogens, but the range of effective exposure times and concentrations was not as large. This may suggest that susceptibility in these experiments was related not only to the effect of copper exposure upon the immune system of fishes but also the interaction of those effects with the specific characteristics of the pathogen.

The specific effects of copper exposure on the immune system of fishes are uncertain. Roales and Perlmutter (1977) demonstrated a reduction in antibody titers to infectious pancreatic necrosis virus and Proteus vulgaris in vaccinated blue gourami (Trichogaster trichopterus) after sublethal exposures to copper. In contrast, Rohovec et al. (1980) have presented data which indicated that a 21-day exposure to sublethal copper concentrations does not affect agglutination antibody titer in coho salmon vaccinated against V. anguillarum. Investigations by Schreck and Lorz (1978) and Donaldson and Dye (1975) showed elevations in serum cortisol levels when salmon were exposed to copper. Increased cortisol concentrations are thought to be related to immunosuppression (Mazeaud et al., 1971).

It is unlikely that an antibody response to V. anguillarum is important in these experiments or those reported by Baker et al. (1980). Since V. anguillarum does not normally occur in freshwater,

the salmonids used in experiments had no contact with the bacterium prior to experimental exposure and sufficient agglutinating antibody would not develop before an epizootic had run its course (Anderson, 1974).

A variety of other stressors have been shown to affect the cellular and humoral immune systems of fishes. Brett (1958) defined stress as the sum of the physiological responses to maintain or re-establish metabolism in the face of a physical or chemical force and Wedmeyer (1970) noted that these metabolic changes often contribute to increased susceptibility to disease. Antibody formation in fishes has been experimentally suppressed by exposure to phenols (Goncharov and Mikryakov, 1970 cited by Anderson, 1974) and crowding (Perlmutter et al., 1973). Robohm and Nitkowski (1974) reported that exposure of fish to 12 mg/l cadmium increased phagocytotic activity but decreased the rate of bacterial destruction while the humoral immunity was unaffected. A reduced number of circulating small lymphocytes resulted from sublethal concentrations of zinc and pulp mill effluent in another investigation (McLeay, 1975). Additionally, Wedemeyer (1970) cited other biochemical effects of stress including lymphopenia, decreased inflammatory response, impaired gamma-globulin formation, and depressed interferon production. Nevertheless, the results presented here for copper stress suggest that stressors do not have a uniform impact on the immune system of the host and other factors

such as species of the host, genetics, age, nutrition, and temperature may be equally important for survival.

Acutely toxic concentrations of copper did not result in increased susceptibility to V. anguillarum. Acute copper toxicity has been reported to cause death as a result of inhibited gas transfer at the gill site (Ellis, 1937), osmoregulatory dysfunction (Cardeilhac et al., 1979), and destruction of neural organs (Gardner and LaRoche, 1973). In several treatments reported here, mortality due to copper ranged from 12.2% to 43.3% (Table 1 and Table 5, Experiment 1) but the final percent mortality due to V. anguillarum was no different from that of the ambient control group. This indicates that, although the physiological consequences of acute copper toxicity resulted in death of part of the population, there was no measureable stress on the immune system of the remaining population which survived that concentration of copper until the time of exposure to the bacteria.

A final consideration concerning differences between results reported here and those of Knittel (1980) and Baker et al. (1979) is methodological. In this investigation fish were transported to a larger raceway for bacterial exposure immediately after copper exposure; Knittel and Baker exposed fish to the pathogen in the aquaria used for copper exposure without moving the fish. Although handling the fish before bacterial exposure was a stressor to fish in both experimental treatments and control groups, it enabled the use of a single dose of

V. anguillarum for all treatments in each replicate. This was useful to minimize variation that might result from exposing fish to separate doses of V. anguillarum while the fish were still in aquaria used for copper treatment.

The effect of handling was examined in two experiments. In one, the percent mortality of a group that was acclimated to the raceways for 96 hours before exposure to the bacterium (handling control) did not differ from that of fish that were transported (ambient control) (Table 5). In the second test (Table 6), the percent mortality of this group (handling control) was significantly ($\alpha = 0.05$) less than that of the ambient control group, but not less than several experimental groups in which fish were also transported. Therefore, it appears that the effect of handling was small and unlikely to mask the effect of copper on susceptibility to V. anguillarum. Other researchers have demonstrated increased susceptibility to pathogens using the same experimental methodology that was used in the experiments reported here (Rohovec et al., 1980; Hetrick et al., 1979). Ideally, methodology could be developed to expose fish to a pathogen through the headbox of a toxicant delivery system thus eliminating the need to handle the fish and assuring a uniform dose of the pathogen.

The impact of intermittent or fluctuating exposures to acute or sublethal toxicant concentrations on fishes or other aquatic life has received little attention in the literature. These effects may be

important because of intermittent industrial discharges or periodic changes of stream flow or effluent discharge regimes. Brooks and Liptak (1979), Zeitoun (1978), and Bass et al. (1977) have investigated the potential impact of intermittent chlorination on aquatic systems. Cairns et al. (1969) reported that bioassay results obtained from exposing three species of fish to toxic concentrations of zinc were similar for both fluctuating and constant input of the toxicant. Sprague (1970) has reviewed several models for the effect of fluctuating toxicant concentrations in which effects may differ from continuous exposure. In one experiment involving sublethal effects of intermittent exposure, Carlson and Herman (1978) showed that diel fluctuations in dissolved oxygen concentration eliminated reproduction of the black crappie (Pomoxis nigromaculatus) acclimated to already low oxygen concentrations. Results reported here did not show an effect of intermittent exposures of copper on susceptibility to V. anguillarum.

Some fish pathogens may be ubiquitous in the water column or may be present in low levels in the resident fish population. Therefore, the potential effects of a toxicant or other adverse environmental conditions in predisposing fish populations to infectious disease should be recognized in water quality management. For example, Aeromonas hydrophila was found to be present in 135 of 147 sites examined across the United States and results showed that it persists under a wide variety of environmental conditions (Hazen et al., 1978). A. hydrophila

lesions in largemouth bass (Micropterus salmoides) have been associated with a thermal effluent (Fliermans et al., 1977) and this pathogen has resulted in major kills in the Southeastern United States (Fliermans et al., 1977; Plumb, 1973; Shotts et al., 1972). Walters and Plumb (1980) reported that low dissolved oxygen concentrations together with high ammonia concentrations and/or high carbon dioxide concentrations increased the susceptibility of channel catfish (Ictalurus punctatus) to intraperitoneally injected A. hydrophila. These investigators also observed an increase in Edwardsiella tarda infections in the experimental fish. Apparently the stress conditions caused naturally occurring latent infections to become manifest.

Nevertheless, the inter-relationship between host, pathogen and environmental stressors is complex and it is difficult to directly apply information about the effects of toxicants on disease susceptibility when establishing specific water quality criteria. The results reported here suggest that all toxicants do not produce a generalized stress response in fishes which lowers their resistance to infectious diseases. Toxicants may promote the infection process of certain types of pathogens to a greater extent than others. In addition, the effect of many toxicants such as organic compounds and chlorine on susceptibility of fish to disease remains largely untested. Perhaps the most useful information in predicting the effect of a toxicant would be an understanding of the mechanisms by which it influences resistance to disease.

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