Appendix H. Analyses of Chronic Data

The following pages contain figures and other information related to the regression and probability distribution analyses that were performed to calculate chronic EC20s. The initial parameter estimates are shown in the tables below. In the figures that follow, circles denote measured responses and solid lines denote estimated regression lines.

Probability Distribution Analysis

Species	Study	Test	Endpoint	Initial Estimates				
				Control Value	EC50	Standard Deviation	EC20	EC10
Snail, *Campeloma decisum (Test 1)*	Arthur and Leonard 1970	LC	Survival	0.925	14.50	0.192	8.73	7.01
Snail, *Campeloma decisum (Test 2)*	Arthur and Leonard 1970	LC	Survival	0.875	11.80	0.339	10.94	9.16
Cladoceran, *Ceriodaphnia dubia (Cinch River)*	Belanger et al. 1989	LC	Reproduction	16.60	33.6	1.15	19.36	14.03
Cladoceran, *Daphnia pulex*	Winner 1985	LC	Survival	1.00	4.57	0.260	2.83	2.24
Cladoceran, *Daphnia pulex*	Winner 1985	LC	Survival	0.900	11.3	0.111	9.16	8.28
Caddisfly, *Clistoronia magnifica*	Nebeker et al. 1984b	LC	Emergence (adult 1st gen)	0.750	20.0	0.300	7.67	5.63
Bluegill (larval), *Lepomis macrochirus*	Benoit 1975	ELS	Survival	0.880	39.8	0.250	27.15	21.60

Logistic Regression Analysis

Species	Study	Test	Endpoint	Initial Estimates				
				Control Value	EC50	Slope	EC20	EC10
Cladoceran, *Ceriodaphnia dubia*	Carlson et al. 1986	LC	Reproduction	13.10	14.6	1.36	9.17	7.28
Cladoceran, *Daphnia magna*	Chapman et al. Manuscript	LC	Reproduction	171.5	16.6	1.40	12.58	10.63
Cladoceran, *Daphnia magna*	Chapman et al. Manuscript	LC	Reproduction	192.1	28.4	1.59	19.89	16.34
Cladoceran, *Daphnia magna*	Chapman et al. Manuscript	LC	Reproduction	88.0	15.8	1.00	6.06	3.64
Rainbow trout, *Oncorhynchus mykiss*	Seim et al. 1984	ELS	Biomass	137.6	40.7	1.69	27.77	22.16
Rainbow trout, *Oncorhynchus mykiss*	Besser et al. 2001	ELS	Biomass	1224	29.2	1.99	20.32	16.74
Chinook salmon, *Oncorhynchus tshawytscha*	Chapman 1975, 1982	ELS	Biomass	0.901	9.55	1.27	5.92	4.47
Fathead minnow, *Pimephales promelas*	Lind et al. manuscript	ELS	Biomass	108.4	11.4	4.00	9.38	8.67

Evaluation of the Chronic Data Available for Freshwater Species

Following is a species-by-species discussion of each chronic test on copper evaluated for this document. Also presented are the results of regression analysis and probability distribution analysis of each dataset that was from an acceptable chronic test and contained sufficient acceptable data. For each such dataset, this appendix contains a figure that presents the data and regression/probability distribution line.

Brachionus calyciflorus. The chronic toxicity of copper was ascertained in 4-day renewal tests conducted at regular intervals throughout the life of the freshwater rotifer, *B. calyciflorus* (Janssen et al. 1994). The goal of this study was to develop and examine the use of this rotifer as a viable test organism. The effect of copper on the age-specific survivorship and fertility of *B. calyciflorus* was determined, but no individual replicate data were provided and only three copper concentrations were tested, which precludes these data from further regression analysis. Chronic limits based on the intrinsic rate of natural increase were 2.5 µg/L total copper (NOAEC) and 5.0 µg/L total copper (LOAEC). The chronic value determined via traditional hypothesis testing is 3.54 µg/L total copper (Table 2a).

Campeloma decisum. Adult *C. campeloma* were exposed to five concentrations of total copper and a control (Lake Superior water) under flow-through conditions in two 6-week studies conducted by Arthur and Leonard (1970). Adult survival in the two separate chronic copper toxicity test trials was markedly reduced in the two highest copper concentrations, 14.8 and 28.0 µg/L, respectively. The authors reported that growth, as determined from cast exoskeleton, was not measurable for this test species, although the authors did observe that the adult snails would not consume food at the two highest copper concentrations. Control survival was 80 percent or greater. Chronic values of 10.88 µg/L total copper were obtained for survival based on the geometric mean of the NOAEC and LOAEC of 8.0 and 14.8 µg/L, respectively, in both tests. The corresponding EC20s were 8.73 and 10.94 µg/L (Table 2a).

Ceriodaphnia dubia. The chronic toxicity of copper to *C. dubia* was determined in ambient river water collected upstream of known point-source discharges of domestic and industrial wastes as part of a water effect ratio study (Carlson et al. 1986). In this study, survival and young production of *C. dubia* were assessed using a 7-day life-cycle test. Organisms were not affected at total copper concentrations ranging from 3 to 12 µg/L (5 to 10 µg/L dissolved copper). There was a 62.7 percent reduction in survival and 97 percent reduction in the mean number of young produced per female at 32 µg/L total copper (27 µg/L dissolved copper). No daphnids survived to produce young at 91 µg/L total copper. Control survival during the study was 80 percent, which included one male. The chronic value EC20 selected for *C. dubia* in this study, 9.17 µg/L derived from a nonlinear regression evaluation, was based on mean number of young produced (reproduction).

The effects of water hardness on the chronic toxicity of copper to *C. dubia* were assessed by Belanger et al. (1989) using 7-day life-cycle tests. *C. dubia* 2 to 8 hours old were exposed to copper in ambient surface water from the New and Clinch Rivers, Virginia. Mean water hardness levels were 179 and 94 mg/L as $CaCO_3$, respectively. Test water was renewed on days 3 and 5. The corresponding chronic values for reproduction based on the NOAEC and LOAEC approach were 7.9 and <19.3 µg/L dissolved copper, respectively. The EC20 value for number of young (neonates) produced in Clinch River water (water hardness of 94 mg/L as $CaCO_3$) was 19.36 µg/L dissolved copper. The EC20 for young produced in New River water was not calculated. The chronic values were converted to total copper using the freshwater conversion factor for copper 0.96 (e.g., 7.897/0.96). The resulting total chronic values for the New and Clinch rivers are 8.23 and 20.17 µg/L, respectively.

Copper was one of 12 toxicants examined by Oris et al. (1991) in their comparisons between a 4-day survival and reproduction toxicity test utilizing *C. dubia* and a standard 7-day life-cycle test for the species. The reported 7-day chronic values for survival and reproduction (mean total young per living female) in two tests based on the traditional hypothesis testing techniques were 24.5 and 34.6 µg/L total copper. Comparable point estimates for these 7-day tests could not be calculated using regression analysis.

Daphnia magna. Blaylock et al. (1985) reported the average numbers of young produced for six broods of *D. magna* in a 14-day chronic exposure to copper. A significant reduction was observed in the mean number of young per female at a concentration of 30 µg/L total copper, the highest copper concentration tested. At this concentration, young were not produced at brood intervals 5 and 6. Reproduction was not affected at 10 µg/L total copper. The chronic value determined for this study (17.32 µg/L total copper) was based on the geometric mean of the NOAEC, 10 µg/L, and LOAEC, 30 µg/L.

Van Leeuwen et al. (1988) conducted a standard 21-day life-cycle test with *D. magna*. The water hardness was 225 mg/L as $CaCO_3$. Carapace length was significantly reduced at 36.8 µg/L total copper, although survival was 100 percent at this concentration. Carapace length was not affected at 12.6 µg/L total copper. No daphnids survived at 110 µg/L concentration. The highest concentration not significantly different from the control for survival was 36.8 µg/L. The lowest concentration significantly different from the control based on survival was 110 µg/L, resulting in a chronic value of 63.6 µg/L for survival. The chronic value based on carapace length was 21.50 µg/L. The 21-day EC10 as reported by the author was 5.9 µg/L total copper.

Chronic (21-day) renewal toxicity tests were conducted using *D. magna* to determine the relationship between water hardness (nominal values of 50, 100, and 200 mg/L as $CaCO_3$, respectively) and the toxicity of total copper (Chapman et al. unpublished manuscript). All test daphnids were <1 day old at the start of the tests. The dilution water was well water from the Western Fish Toxicology Station (WFTS), Corvallis, Oregon. Test endpoints were reproduction (total and live young produced per female) and adult survival. The survival of control animals was 100 percent at nominal water hardness levels of 50 and 200 mg/L as $CaCO_3$, and 80 percent at a hardness of 100 mg/L as $CaCO_3$. The chronic values for total young produced per female (fecundity) based on the geometric mean of the NOAEC and LOAEC were 13.63, 29.33, and 9.53 µg/L at the nominal hardness levels of 50, 100, and 200 mg/L as $CaCO_3$, respectively. The corresponding EC20 values for reproduction calculated using nonlinear regression analysis were 12.58, 19.89, and 6.06 µg/L total copper. The chronic toxicity of copper to *D. magna* was somewhat ameliorated from an increase in water hardness from 50 to 100 mg/L as $CaCO_3$, but slightly increased from 100 to 200 mg/L as $CaCO_3$.

Daphnia pulex. Winner (1985) evaluated the effects of water hardness and humic acid on the chronic toxicity (42-day) of copper to *D. pulex*. Contrary to the expectation that sublethal endpoints are more sensitive indicators of chronic toxicity, reproduction was not a sensitive indicator of copper stress in this species. Water hardness also had little effect on the chronic toxicity of copper (similar to *D. magna* trends), but humic acid significantly reduced chronic toxicity of copper when added to the varying water types. The survival chronic values based on the NOAEC and LOAEC values for the three low to no humic acid studies were 4.90, 7.07, and 12.25 µg/L total copper at hardnesses of 57.5, 115, and 230 (0.15 mg/L HA) µg/L as $CaCO_3$, respectively. The EC20 values calculated for the low and high hardness studies using nonlinear regression techniques were 2.83 and 9.16 µg/L at hardness values of 57.5 and 230 (0.15 mg/L HA) µg/L as $CaCO_3$, respectively.

Clistoronia magnifica. The effects of copper on the lifecycle of the caddisfly, *C. magnifica*, were examined in Nebeker et al. (1984b). The test included continuous exposure of first-generation aquatic larvae and pupae through to a third generation of larvae. A significant reduction in adult emergence occurred at 13.0 µg/L total copper from first-generation larvae. No observed adverse effect to adult emergence occurred at 8.3 µg/L total copper. Percent larval survival was close to the control value of 80 percent. The chronic value based on hypothesis testing was 10.39 µg/L total copper. The corresponding EC20 value for adult emergence was 7.67 µg/L total copper.

Oncorhynchus mykiss. The growth and survival of developing *O. mykiss* embryos continuously and intermittently exposed to copper for up to 85 days post-fertilization was examined by Seim et al. (1984). Results only from the continuous exposure study are considered here for deriving a chronic value. A flow-through apparatus was used to deliver six concentrations and a control (untreated well water; average of 3 µg/L copper) to a single incubation chamber. Continuous copper exposure of steelhead embryos in the incubation chambers was begun 6 days post-fertilization. At 7 weeks post-fertilization, when all control fish had hatched and reached swim-up stage, subsamples of approximately 100 alevins were transferred to aquaria and the same exposure pattern continued. Dissolved oxygen remained near saturation throughout the study. Water hardness averaged 120 mg/L as $CaCO_3$. Survival of steelhead embryos and alevins exposed continuously to total copper concentrations in the range of 3 (controls) to 30 µg/L was greater than 90 percent or greater. Survival was reduced at 57 µg/L and completely inhibited at 121 µg/L. A similar effect on survival was observed for embryos and alevins exposed to a mean of 51 (peak 263) and 109 (peak 465) µg/L of copper in the intermittent exposure, respectively. The adverse effect of continuous copper exposure on growth (measured on a dry weight basis) was observed at concentrations as low as 30 µg/L. (There was a 30 percent reduction in growth during the intermittent exposure at 16 µg/L.) The chronic limits for survival of embryos and alevin steelhead trout exposed continuously to copper were 16 and 31 µg/L, respectively (geometric mean = 22.27 µg/L). The EC20 for biomass for the continuous exposure was 27.77 µg/L.

Besser et al. (2001) conducted an ELS toxicity test with copper and the rainbow trout, *O. mykiss*, starting with eyed embryos and continuing for 30 days after the fish reached the swim-up stage. The total test period was 58 days. The test was conducted in ASTM moderately hard reconstituted water with a hardness of approximately 160 to 180 mg/L as $CaCO_3$. Twenty-five eyed embryos were held in each of four replicate egg cups at each concentration. Survival was monitored daily. At the end of the test, surviving fish in each replicate chamber were weighed (dry weight). Dry weights were used to determine growth and biomass of surviving fish. The no observed effect concentrations (NOECs) for survival and biomass were both 12 µg/L and the lowest observed effect concentrations (LOECs) for survival and biomass was also the same for both endpoints, 22 µg/L. The chronic values for biomass and survival based on the geometric mean of the NOEC and LOEC were 16.25 µg/L. The corresponding EC20 for biomass was 20.32 µg/L.

Oncorhynchus tshawytscha. The draft manuscript prepared by Chapman (1975/1982) provides the results from a 4-month egg through fry partial chronic test conducted to determine the effects of copper on survival and growth of *O. tshawytscha*. Continuous exposure occurred from several hours post-fertilization through hatch, swim-up, and feeding fry stages. The test was terminated after 14 weeks post-hatch. The dilution water was WFTS well water. Because of the influence of the nearby Willamette River on the hardness of this well water, reverse osmosis water was mixed periodically with ambient well water to attain a consistent hardness. The typical hardness of this well water was approximately 23 mg/L as $CaCO_3$. Control survival exceeded 90 percent for the test. The measured total copper concentrations during the test were 1.2 (control), 7.4, 9.4, 11.7, 15.5, and 20.2 µg/L, respectively. Copper adversely affected survival at 11.7 µg/L copper and higher, and growth was reduced at all copper concentrations tested compared with the growth of control fish. The chronic limits for copper in this study were estimated to be less than 7.4 µg/L. The EC20 value estimated for biomass is 5.92 µg/L total copper based on a logistic nonlinear regression model.

Salmo trutta. McKim et al. (1978) examined the survival and growth (expressed as standing crop) of embryo-larval and early juvenile brown trout to copper. The most sensitive exposure was with embryos exposed for 72 days. The NOAEC and LOAEC, as obtained from the figure, were 20.8 and 43.8 µg/L total copper, respectively. Data were not available to calculate point estimates at the 20 percent effect level using regression analysis. The chronic value selected for this species was 29.91 µg/L total copper (geometric mean of 20.8 and 43.8 µg/L total copper).

Salvelinus fontinalis. Sauter et al. (1976) examined the effects of copper on selected freshwater fish species at different hardness levels (softwater at 37.5 mg/L as $CaCO_3$; hardwater at 187 mg/L as $CaCO_3$) during a series of partial life-cycle (PLC) tests. The species tested were brook trout (*Salvelinus fontinalis*), channel catfish (*Ictalurus punctatus*), and walleye (*Stizostedion vitreum*). Because of the poor embryo and larval survival of control animals (in all cases less than 70 percent), results from tests with channel catfish and walleye were not included in Table 2a. One of the replicate control chambers from the PLC tests conducted with brook trout in hard water also exhibited poor hatchability (48 percent) and survival (58 percent) between 31 and 60 days of exposure. Therefore, the data for brook trout in hard water were not included in the subsequent EC20 (regression) analysis either.

The softwater test with brook trout was conducted using untreated well water with an average water hardness of 35 mg/L as $CaCO_3$. This PLC exposure consisted of six copper concentrations and a control. Hatchability was determined by examining randomly selected groups of 100 eggs from each replicate exposure tank. Growth and survival of fry were determined by impartially reducing the total sample size to 50 fry per tank and assessing their progress over 30 day intervals up to 60 days post-hatch. The chronic limits based on the growth (wet weight and total length) of larval brook trout after 60 days of exposure to copper in soft water were <5 and 5 µg/L. The resultant chronic value for soft water based on hypothesis testing was <5 µg/L. The corresponding EC20 values based on total length, wet weight, and biomass (the product of wet weight and survival) for brook trout in the soft-water exposures after 60 days were not amenable to nonlinear regression analysis.

McKim et al. (1978) examined survival and growth (expressed as standing crop) of embryo-larval and early juvenile brook trout exposed to copper. The embryo exposure was for 16 days, and the larval-early-juveniles exposure lasted 60 days. The NOAEC and LOAEC were 22.3 and 43.5 µg/L total copper, respectively. Data were not available to calculate point estimates at the 20 percent effect level using regression analysis. The chronic value for this species was 31.15 µg/L total copper (geometric mean of 22.3 and 43.5 µg/L total copper).

Salvelinus namaycush. McKim et al. (1978) examined the survival and growth (expressed as standing crop) of embryo-larval and early juvenile lake trout exposed to copper. The embryo exposure was for 27 days, and the larval-early-juveniles exposure lasted 66 days. The NOAEC and LOAEC were 22.0 and 43.5 µg/L total copper, respectively. Data were not available to calculate point estimates at the 20 percent effect level using regression analysis. The chronic value for this species was 30.94 µg/L total copper (geometric mean of 22.0 and 43.5 µg/L total copper).

Esox lucius. McKim et al. (1978) examined the survival and growth (expressed as standing crop) of embryo-larval and early juvenile northern pike exposed to copper. The embryo exposure was for 6 days, and the larval-early-juveniles exposure lasted 34 days. The NOAEC and LOAEC were 34.9 and 104.4 µg/L total copper, respectively. The authors attributed the higher tolerance of *E. lucius* to copper to the very short embryonic exposure period compared with salmonids and white sucker, *Catostomus* *commersoni*. Data were not available to calculate point estimates at the 20 percent effect level using regression analysis. The chronic value for this species was 60.36 µg/L total copper (geometric mean of 34.9 and 104.4 µg/L total copper).

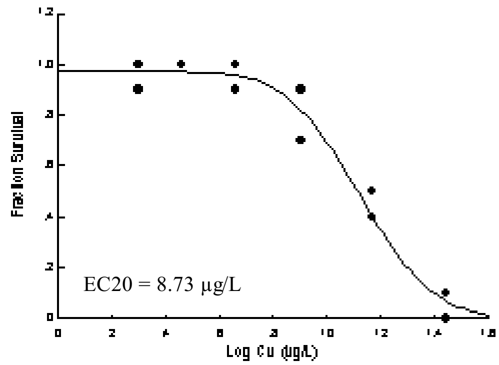
Pimephales notatus. An experimental design similar to that described by Mount and Stephan (1967) and Mount (1968) was used to examine the chronic effect of copper on the bluntnose minnow, *P. notatus* (Horning and Neiheisel 1979). Measured total copper concentrations were 4.3 (control), 18.0, 29.9, 44.1, 71.8, and 119.4 µg/L, respectively. The experimental dilution water was a mixture of spring water and demineralized City of Cincinnati tap water. Dissolved oxygen was kept at 5.9 mg/L or greater throughout the test. Total water hardness ranged from 172 to 230 mg/L as $CaCO_3$. The test was initiated with 22 6-week-old fry. The fish were later separated according to sex and thinned to a sex ratio of 5 males and 10 females per duplicated test chamber. Growth (total length) was significantly reduced in parental and first (F_1) generation *P. notatus* after 60 days of exposure to the highest concentration of copper tested (119.4 µg/L). Survival of parental *P. notatus* exposed to this same high test concentration was also lower (87 percent) at the end of the test compared with the other concentrations (range of 93 to 100 percent). Copper at concentrations of 18 µg/L and greater significantly reduced the number of eggs produced per female. The number of females available to reproduce was generally the same up to about 29.9 µg/L of copper. The chronic limits were based on an NOAEC and LOAEC of <18 and 18 µg/L for number of eggs produced per female. An EC20 was not estimated by nonlinear regression; nevertheless, in this case an EC20 is likely to be substantially below 18 µg/L.

Pimephales promelas. The results from a 30-day ELS toxicity test to determine the chronic toxicity of copper to *P. promelas* using dilution water from Lake Superior (hardness ranging from 40 to 50 mg/L as $CaCO_3$) was included in Table 2a from a manuscript prepared by Lind et al. in 1978. In this experiment, five test concentrations and a control were supplied by a continuous-flow diluter. The exposure began with embryos 1 day post-fertilization. Pooled results from fish dosed in replicate exposure chambers were given for mean percentage embryo survival to hatch, mean percentage fish survival after hatch, and mean fish wet weight after 30 days. The percentage of embryo survival to hatch was not affected by total copper concentrations as high as 52.1 µg/L total copper. Survival after hatch, however, was compromised at 26.2 µg/L, and mean wet weight of juvenile fathead minnows was significantly reduced at 13.1 µg/L of copper. The estimated EC20 value for biomass was 9.376 µg/L total copper.

Catastomus commersoni. McKim et al. (1978) examined the survival and growth (expressed as standing crop) of embryo-larval and early juvenile white sucker exposed to copper. The embryo exposure was for 13 days, and the larval-early-juvenile exposure lasted 27 days. The NOAEC and LOAEC were 12.9 and 33.8 µg/L total copper, respectively. The resulting chronic value based on hypothesis testing for this species was 20.88 µg/L total copper (geometric mean of 12.9 and 33.8 µg/L total copper).

Lepomis macrochirus. Results from a 22-month copper life-cycle toxicity test with bluegill (*L. macrochirus*) were reported by Benoit (1975). The study included a 90-day embryo-larval survival and growth component. The tests were conducted at the U.S. EPA National Water Quality Laboratory in Duluth, Minnesota, using Lake Superior water as the dilution water (average water hardness = 45 mg/L as $CaCO_3$). The test was initiated in December 1969 with 2-year-old juvenile *L. macrochirus*. In May 1971, the fish were sexed and randomly reduced to three males and seven females per tank. Spawning commenced on 10 June 1971. The 90-day embryo-larval exposure was initiated when 12 lots of 50 newly hatched larvae from one of the two control groups were randomly selected and transferred to duplicate grow-out chambers at 1 of 6 total copper concentrations: 3 (control), 12, 21, 40, 77, and 162 µg/L, respectively. In the 22-month juvenile through adult exposure, survival, growth, and reproduction were unaffected at 77 µg/L of copper and below. No spawning occurred at 162 µg/L. Embryo hatchability and survival of 4-day-old larvae at 77 µg/L did not differ significantly from those of controls. However, after 90 days of exposure, survival of larval *L. macrochirus* at 40 and 77 µg/L was significantly lower than for controls, and no larvae survived at 162 µg/L. Growth remained unaffected at 77 µg/L. Based on the 90-day survival of bluegill larvae, the chronic limits were estimated to be 21 and 40 µg/L (geometric mean = 28.98 µg/L). The corresponding EC20 for embryo-larval survival was 27.15 µg/L.

***Campeloma decisum* (Test 1), Life-cycle, Arthur and Leonard 1970**



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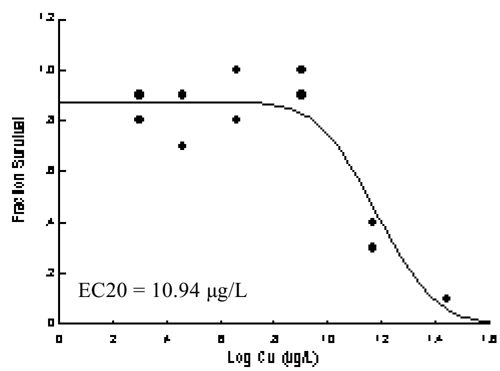
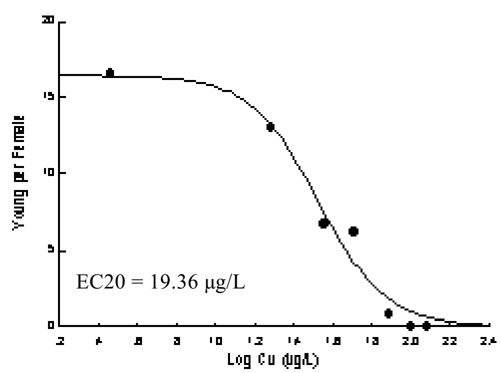
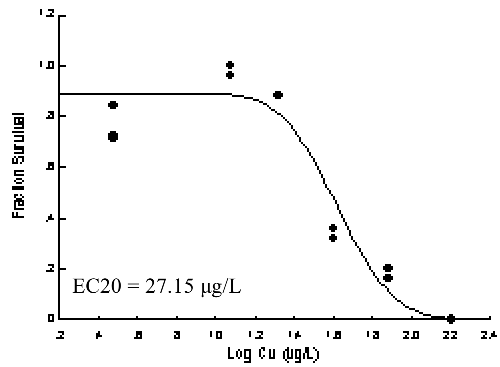


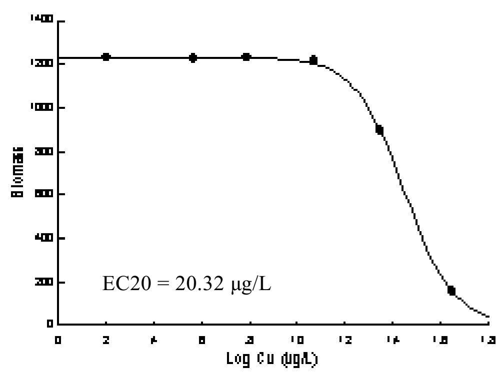
***Ceriodaphnia dubia* (Clinch River), Life-cycle, Belanger et al. 1989**



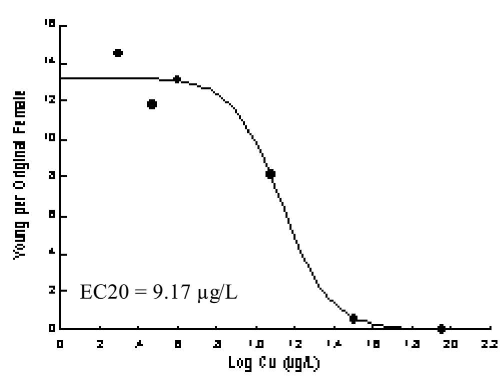
***Lepomis macrochirus*, Early Life-stage, Benoit 1975**



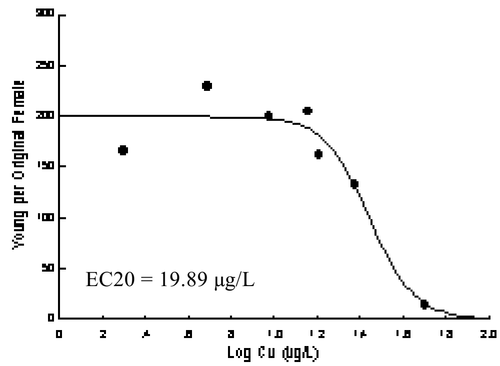
***Oncorhynchus mykiss*, Early Life-Stage, Besser et al. 2001**



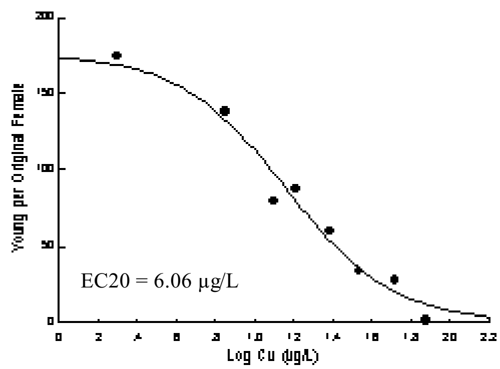
***Ceriodaphnia dubia*, Life-cycle, Carlson et al. 1986**



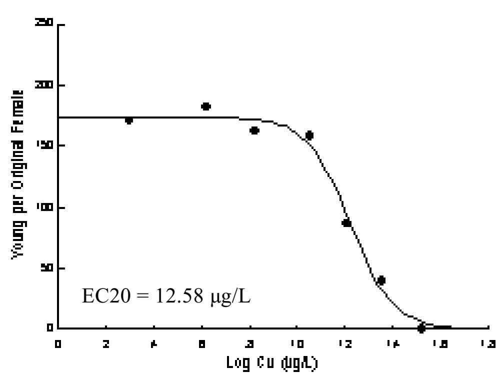
Daphnia magna **(Hardness 104), Life-cycle, Chapman et al. Manuscript**



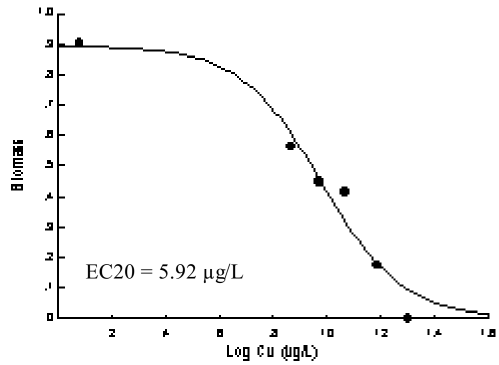
Daphnia magna **(Hardness 211), Life-cycle, Chapman et al. Manuscript**



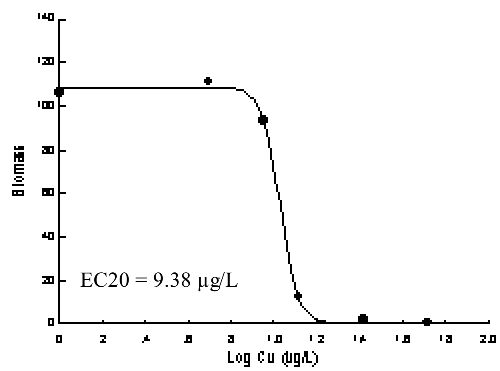
Daphnia magna **(Hardness 51), Life-cycle, Chapman et al. Manuscript**



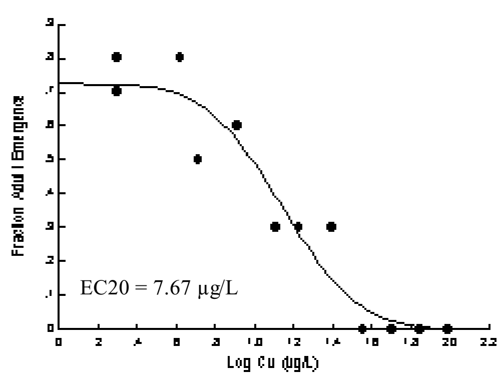
***Oncorhynchus tshawytscha*, Early Life-Stage, Chapman 1975 & 1982**



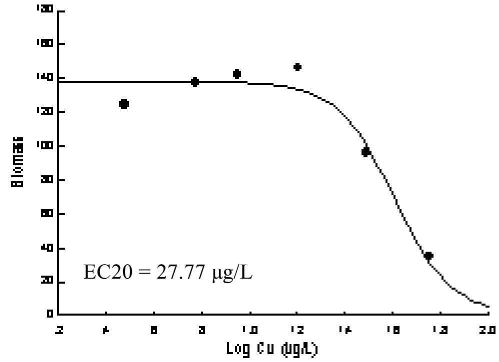
***Pimephales promelas*, Early Life-stage, Lind et al. 1978**



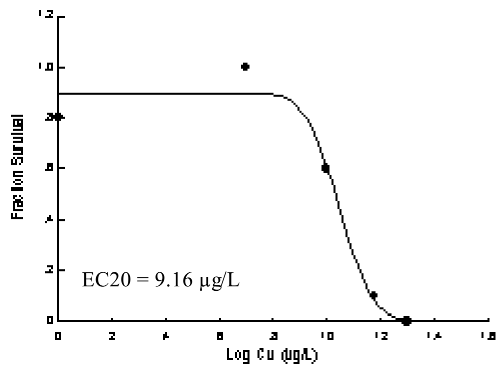
***Clistoronia magnifica*, Life-cycle, Nebeker et al. 1984a**



***Oncorhynchus mykiss,* Early Life-stage, Seim et al. 1984**



***Daphnia pulex* (Hardness 230 HA 0.15), Life-cycle, Winner 1985**



***Daphnia pulex (Hardness 57)*, Life-cycle, Winner 1985**