ACUTE EXPOSURES OF SALMONID EMBRYOS TO TOTAL DISSOLVED SOLIDS

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ACUTE EXPOSURES OF SALMONID EMBRYOS TO TOTAL DISSOLVED SOLIDS

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Abstract

Two exposure methodologies are described here utilizing embryonic and juvenile life stages of several species of salmonids. Specific life stages of the fish were exposed to solutions of varying total dissolved solids (TDS) modeled after the measured produced water from the Red Dog Mine in Kotzebue, Alaska. Embryonic and juvenile coho salmon (*O. kisutch*) were exposed for 96 hours to determine acute response to TDS. Following exposure, fish were grown out to button up to assess delayed effects. Results from the 96-hour study suggest fertilization is the most sensitive developmental stage of salmon exposed to TDS. Six fish species were then used to assess a new 24-hour embryo toxicity study during fertilization. We examined short- and long-term mortality, number of unfertilized eggs, and the overall percent affected. The endpoint for the assay is the success of egg fertilization. Based on the results of these experiments, it is reasonable to conclude that the fertilization assay can be generalized across these species and may be useful in setting site-specific criteria for discharging wastes.

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Chapter 1

Introduction

Total dissolved solids (TDS) are found in every natural water source. TDS is a measure of the total common ions found in a sample of water. Although the concept of TDS is simple, the complexity comes in the assessment of TDS. For example, a TDS solution may be composed of only sodium chloride (NaCl) at a concentration of 2000 mg/L, or a combination of many different ions, with an equivalent TDS concentration as the NaCl solution.

TDS varies widely in its source, toxicity and composition. Some common ions found in nature are calcium, sodium, potassium, magnesium, chloride, and sulfate. These ions may enter the water naturally through surface runoff, subsurface leaching, or erosion. Bodies of water in close proximity, such as an area with many lakes, can have drastically different TDS measurements ranging from 200 mg/L to over 100,000 mg/L (Gosselin 1997). TDS may also enter aquatic systems through anthropogenic sources. In various studies shale oil leachates (Meyer et al 1985), coal pile leachates (Carlson & Carlson 1994), mining effluents (Chapman et al 2000, Kline & Stekoll 2000, Vinyard 1996), and irrigation drain waters (Dickerson et al 1996, Ingersoll et al 1992, Kilbride et al 1998) have been shown to be toxic as a result of increased concentrations of common ions. Studies of municipal landfills (Borden & Yanoschak 1990, Ragle et al 1995, Ross 1990), and mine tailings deposits (Olyphant et al 1991) show that seasonal changes in TDS loading to nearby water sources and groundwater are dependant on levels of rain and overland water flow. Studies of industrial effluents such as open pit mine waters (Whitehead & Macdonald 1998) and longwall mine discharge (Booth & Bertsch 1999)

show increases in TDS due to discharges, but the impacts of these TDS enhanced waters has not been addressed.

TDS from Industrial effluents and leachates, if untreated, can impact watershed systems. Usis and Foote (1991) found that increased levels of TDS due to strip mining upstream of the Stillfork Swamp Nature Preserve, Ohio, were strongly correlated with decreased survival of the wetland caddisfly *Limnephilus indivisus*. The composition of TDS was not addressed in this study, though effects were seen at concentrations of ~800 mg/L and above. Linton et al (1983) found heightened occurrence of deformed cocoons with fewer eggs and embryos in *Nephelopsis obscura* and *Erpobdella punctata* due to high TDS levels. *E. punctata* also showed a decline in the proportion of mature individuals laying cocoons as TDS concentrations tested approached 2000 mg/L. Although there was no significant effect on the number of cocoons produced per individual.

Drainage of water basins for agricultural irrigation and the subsequent return of the irrigation drain waters can increase TDS loads. Contaminants in these waters may pose a threat to fisheries in their areas. Dickerson *et al* (1999) found that current levels of TDS (13000 mg/L composed mainly of calcium, magnesium, sodium, potassium, bicarbonate, carbonate, sulfate and chloride) in Walker Lake, Nevada, adversely affected both the survival and growth of Lahontan cutthroat trout (*Oncorhynchus clarki henshawi).* Any future increase in the TDS load is likely to further decrease survival. Survival was reduced by exposure to all TDS concentrations above the control conditions of 130 mg/L. Dickerson *et al.* found that larger fish are more tolerant of higher

concentrations. They hypothesized that acclimation to the high TDS levels prior to release of the artificially propagated fishes might increase survival rates.

TDS is a vague term for a complex pollutant. It is difficult to set water quality standards for this particular parameter based simply on the concentration of TDS. The term Total Dissolved Solids is defined in two different ways. One definition refers to the filtration and subsequent drying of the filtered water sample where TDS is the mass of the residue left on the desiccation apparatus (American 1995). This can be calculated by the equation: mg total dissolved solids/L = ((A-B)*1000)/sample volume, mL where: A = weight of dried residue + dish in mg and B = weight of dish in mg (American 1995). This equation gives information on the milligrams per liter of dissolved solids in the water, but tells us nothing about the individual components of the mixture. The second way of defining TDS is the sum of the concentrations of specific individual ions in the solution. Total dissolved solids = 0.6 * (alkalinity as $[CaCO_3]$) + $[Na^+]$ + $[K^+]$ + $[Ca^{2+}]$+ $[Mg^{2+}]$+ $[Cl^-]$ + $[SO_4^{2-}]$ + $[SiO_3^{2-}]$ + $[NO_3^-]$ + $[F^-]$ (American 1995). The units used are usually in mg/L. For this second definition one must know or measure the concentrations of individual ions. The second definition gives some information on the ions in a mixture but only includes those listed in the formula. A sample could include other ions (such as Fe^{+3} or phosphate), and have two very different measures of TDS between the two definitions. The first definition would give a higher TDS level than the second because the ions would be a part of the whole mixture, while the second would show a lower TDS measurement because those ions are not included in the formula for the TDS calculation.

The toxicity of the mixture however, is not either evident or predictable from the measures given in either definition and further analysis of the water samples is needed.

The speciation of TDS in a body of water will be important for toxicity determination. Sodium chloride at 2000 mg/L may be harmless to an aquatic organism, while the same concentration of sodium sulfate may prove lethal. Therefore it is not the total TDS that is of interest, but rather the toxic component of the solution. Toxicity of TDS depends on what individual components exist in the TDS mixture and the concentration at which they occur.

Toxicity of fresh waters with high TDS is documented to be dependent on the specific ion composition of the waters (Mount et al 1997). Mount et al (1997) studied the effects of single ions and combinations of those on three standard test organisms (*Ceriodaphnia dubia, Daphnia magna,* and *Pimephales promelas*), showing that combinations of ions produced TDS concentration LC50's much lower than their single ion counterparts. The potassium salts investigated produced the lowest LC50's (580-780 mg/L in *C. dubia*, 440-880 mg/L in *D. magna*, and 750-1,090 mg/L in *P. promelas* respectively) in all three species tested. These species were slightly more tolerant of magnesium (880-1,770 mg/L in *C. dubia* for 24-h exposure), and calcium (1,770-2,680 mg/L in *C. dubia* for 24-h exposure), and the most tolerant of sodium (3,080-3,740 mg/L in *C. dubia* for 24-hour exposure). The use of sulfates in the salts as opposed to chloride lowered LC50 values as well. The actual composition of TDS can influence an organism's response to them. Pollutants may damage organisms with immediate lethal consequences, or with chronic dose effects. In many cases juveniles or early life stages

of organisms are often more sensitive to toxicants than are adults (US EPA 1991). Cleveland *et al.* (1986) found earlier life stages of book trout (embryos and sac-fry or larvae) to be more sensitive to aluminum and pH fluctuation interactions than older individuals (30 days post-hatch buttoned up specimens).

Several organisms have been utilized as test species for TDS toxicity. Some species commonly used as indicators of toxicity are: daphnia (Chapman et al 2000, Dickerson et al 1996), minnows (Dickerson et al 1996, Mount et al 1997), and salmonids (Alsop & Wood 1999, Arkoosh et al 1998, Ingersoll et al 1990, Kocan & Landolt 1989, Chapman et al 2000). Ketola et al (1988) found that salmonid embryos exposed to high concentrations of calcium (520 mg/L or greater) during water-hardening decreased survival rates of several salmonid species. Chapman et al (2000) exposed both chironomid (*Chironomus tentans*) larvae and embryonic and juvenile rainbow trout (*Oncorhynchus mykiss*) to two synthetic TDS mixtures modeled after the ionic composition of two mine effluents from Alaskan mining operations. Both life stages of rainbow trout were unaffected by acute exposure to concentrations of TDS up to 2000 mg/L, however, the chironomid larvae showed effects at concentrations above 1100 mg/l. If wild juvenile rainbow trout feed upon chironomids, mortality of this food source could impact production of the salmonid population leading to system wide impacts from smaller adult populations. Decreased salmon returns will, in turn, reduce the input of nutrients (marine-derived) into the natal stream and adjoining riparian habitats (Wipfli et al 1998).

Salmonids have been widely utilized in toxicology studies. Salmonids are an indigenous species found throughout the freshwater ecosystems of Alaska. The culturing of salmonids, their development, and life history has been extensively documented in literature. Toxicity studies with salmon include those involving acid/pH fluctuation (Brown 1981, Ingersoll et al 1990), dissolved solids (Chapman et al 2000, Ketola et al 1987, Ketola et al 1988), herbicides (Wan et al 1992), hydrocarbons (Arkoosh et al 1998, Heintz et al 1999), and metals (Alsop & Wood 1999, Cleveland et al 1986, Lorz et al 1978, Finlayson & Verrue 1982, Guadagnolo et al 2000, Mount et al 1990, Zitko and Carson 1976). Embryos of pacific salmon have been shown to be physiologically sensitive to pollutants, and can be adversely affected in later life, or even in later generations, by exposure as embryos to sublethal concentrations of pollutants (Heintz et al 1999). Salmon spawn in bodies of freshwater. Anadromous salmonids will reside within these lakes, rivers or streams for as long as two years as juveniles prior to downstream migration to the oceans, making them an ideal test species for effluent toxicity tests. They are a likely species to be impacted by industrial effluent outflow or runoff during their early life history.

Many produced waters tend to be very high in TDS (Pillard et al 1996). Discharge of pollutants or any produced waters into the environment requires a National Pollutant Discharge Elimination System (NPDES) permit. In Alaska the charge falls on the permittee to show that the discharge water causes no chronic toxicity to receiving waters or aquatic biota. TDS discharge is permitted on a site-by-site basis. The current water quality parameters regarding discharge limits are aimed at the use or potential uses

of the water such as potable water or water for aquaculture. In Alaska discharges must cause no chronic toxicity to organisms (Alaska 1999). Acute toxicity is not addressed. The water quality guidelines associated with this permitting process should be made stringent enough to ensure survival of indigenous species while at the same time not being so stringent as to be prohibitively expensive to achieve, causing industrial activities to cease.

While there are assays available to test industrial effluents and their impacts on a water receiving system (Duodoroff et al 1951, U.S. EPA 1991), the assays may not be applicable to indigenous species of an area. In order to write site-specific criteria regarding discharge of industrial effluents to receiving water, applicable assays must be run using the site-specific effluent or a synthetic mock-up of that effluent and site-specific species. This fact was illustrated by LeBlond and Duffy (2001) who found that Microtox® and *Selenastrum capricornatum* assays with a non-site-specific synthetic TDS mixture cannot predict the toxic effects of a discharged effluent.

We have seen evidence that TDS toxicity is dependent upon its composition and the concentration of individual ions. Toxicity can't be predicted simply on the basis of a TDS measurement in mg/L. In addition, while TDS occurs naturally in watershed systems, it is the anthropogenic sources of TDS that are cause for concern. But because TDS is a rather complex or variable pollutant, it is difficult to regulate. As industrially created discharges with elevated levels of TDS have been show to be toxic, there is a need for more research on TDS toxicity in order to set meaningful regulatory standards. This research should include testing that utilizes species, and life stages of these species,

environmentally relevant to the area being impacted. This research should also develop a methodology that produces results able to aid in setting meaningful regulatory standards.

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Chapter 2

Acute Exposures of Salmonid Embryos to Total Dissolved Solids[1]

[1] Failor-Rounds, B. J. 2003. Acute Exposures of Salmonid Embryos to Total Dissolved Solids. Prepared for submission in Transactions of the American Fisheries Society.

Abstract

Coho salmon (*O. kisutch*) were exposed to model solutions of varying total dissolved solids (TDS) during several life stages of development from fertilization through button-up. The TDS solution was modeled after the measured produced water from the Red Dog Mine in Kotzebue, Alaska. Embryos and various stages of development of juveniles were exposed for 96 hours to determine acute response to TDS. Following the 96 hour exposures, the fish were grown out to button up to assess delayed effects from exposure. We looked at four separate aspects as a result of the exposures at fertilization: short-term mortality, long-term mortality, the number of unfertilized eggs, and the overall percent affected. Exposures at the other developmental stages were assessed for both short term and delayed mortalities. Effects were dependent on the stage of development and the manner in which the fish were exposed. Results suggest that fertilization is the most sensitive of the developmental stages of salmon exposed to TDS.

Introduction

Total dissolved solids (TDS) are commonly found in industrial effluents and exist in natural water bodies. TDS is defined as the residue left in a vessel following the filtering, evaporation and subsequent drying of a sample in an oven at a defined temperature (Franson 1995, Piper *et al.* 1986). Natural waters can have a wide range (10-100,000 ppm) in TDS content (Baulding 1976, Bricker & Jones 1995, Dickerson & Vinyard 1999).

Many produced waters tend to be very high in TDS (Pillard *et al.* 1996). High total dissolved solids levels have been observed in leachates of mined raw oil shale (Meyer *et al.* 1985), gold mining effluents (Kline & Stekoll 2000), solid waste landfill leachates (Ragle *et al.* 1995), and irrigation drain waters (Ingersoll *et al.* 1992). Discharge of pollutants or any produced waters into the environment requires a National Pollutant Discharge Elimination System (NPDES) permit. In Alaska water quality standards regarding TDS are based on the proposed use of the water. TDS limits are often set on a site-specific basis (Mary Kate McKearney pers. comm.).

The chemical composition and toxicity of a water sample are not well defined by the simple measure of TDS. Toxicity of fresh waters with high TDS is documented to be dependent on the specific ion composition of the waters (Mount *et al.* 1997). Mount *et al.* (1997) studied the effects of single ions and combinations of ions on three standard test organisms (*Ceriodaphnia dubia, Daphnia magna,* and *Pimephales promelas*). Their results showed that combinations of ions produced LC50's much lower than their single ion counterparts. The actual composition of TDS can influence an organism's response

to TDS exposure. Pollutants may damage organisms with immediate lethal consequences, or with chronic dose effects.

Little research has been done regarding the toxicity of TDS to aquatic organisms (Chapman *et al.* 2000, Dickerson & Vinyard 1999, Kline & Stekoll 2000). Chapman *et al.* (2000) have investigated the effects of TDS on daphnia and early life stages of rainbow trout. However, they did not expose all the life stages of these trout. Specific life stages of an organism may be most susceptible to a toxicant and an important determinant of sensitivity. In many cases juveniles or early life stages of organisms are often more sensitive to toxicants than are adults (Weber ed 1991). Some species commonly used as indicators of toxicity are: daphnia (Chapman *et al.* 2000, Dickerson *et al* 1996), minnows (Dickerson *et al.* 1996, Mount *et al.* 1997), and salmonids (Alsop & Wood 1999, Arkoosh *et al.* 1998, Ingersoll *et al.* 1990, Kocan & Landolt 1989, Chapman *et al.* 2000). Ideally it would be optimal to test a variety of species when assessing the toxicity of a water sample. But in practice it is often necessary to select a surrogate species. In this paper we have used salmon as a representative fish species.

Salmonid species are indigenous to Alaska and are found throughout its freshwater ecosystems. Their development and life history have been extensively documented in literature (Ballard 1973, Groot & Margolis 1998, Velsen 1980). Salmonids have been widely utilized in toxicological studies, including those involving acid/pH fluctuation (Brown 1981, Ingersoll *et al.* 1990), dissolved solids (Chapman *et al.* 2000, Ketola *et al.* 1987, Ketola *et al.* 1988), herbicides (Wan *et al.* 1992), hydrocarbons (Arkoosh *et al.* 1998, Heintz *et al.* 1999), and metals (Alsop & Wood 1999, Cleveland *et*

al. 1986, Lorz *et al.* 1978, Finlayson & Verrue 1982, Guadagnolo *et al.* 2000, Mount *et al.* 1990, Zitko and Carson 1976). Embryos of pacific salmon have been shown to be physiologically sensitive to pollutants. Exposure can cause adverse effects in later life, or even in later generations, by exposure as embryos to sublethal concentrations of pollutants (Heintz *et al.* 1999). Because salmonids spawn in fresh water they are likely to be impacted by industrial effluent outflow or runoff. In addition anadromous salmonids will reside in lakes, rivers or streams for as long as two years as juveniles. Therefore, salmonids are ideal test organisms for effluent toxicity tests.

The objective of this research was to determine if certain stages of the life cycle of salmonids are more sensitive to exposure to TDS, and if any delayed effects are associated with such exposure. To achieve this goal it was necessary to develop an acute bioassay on the effects of TDS on the life stages of salmonids. The results of the research may provide baseline information for setting regulations to insure that TDS mixtures entering the aquatic ecosystem cause no adverse effects to aquatic life.

Methods

The experimental animals used were coho (*Oncorhynchus kisutch*) salmon. Broodstock were obtained as mature fish returning to Douglas Island Pink and Chum Inc.'s (DIPAC) Macaulay Hatchery located in Juneau, Alaska. Research took place in the university wet lab area in the Macaulay Hatchery.

Ten female and ten male coho salmon (*O. kisutch*) were used in the bioassays. Females were spawned (Piper *et al.* 1986) into separate 1-gallon Ziploc® bags. The bags

were then sealed and placed into a cooler for transportation to the wet-lab area. Males were spawned (Piper *et al.* 1986) separately into Dixie® cups to monitor quality (excessive blood or feces in the milt caused us to throw out the sample and take another male). The cups were then placed in separate one-quart Ziploc® bags and placed in the cooler for transportation to the wet-lab area. In the wet-lab, the eggs were pooled in a disinfected plastic bowl, and the milt was pooled in a disinfected 200-mL plastic cup.

Ninety-six hour bioassays were performed at the pre-determined benchmark stages of fertilization, epiboly, eyed, hatch, and button-up. The 2000-broodyear series also included an assay on embryos at the stage between fertilization and epiboly (BEF). For the 96-hour exposure at fertilization, approximately 30 eggs were placed in a cup containing 100 mL of solution. Milt (0.2 mL) was added to the cup with a syringe. The cup was filled (another 100 mL) to facilitate mixing of the milt and eggs. Eggs were allowed to sit for two minutes and then rinsed until the rinse was clear (usually two rinses). Different methods of exposing the eggs during and just after fertilization were employed. The Concentration-Concentration treatment consisted of eggs fertilized in the test (TDS) solution for two minutes and transferred to the same concentration of test solution for the remainder of the assay. The Concentration-Freshwater treatment consisted of eggs fertilized in the test solution for two minutes and then transferred to control water. In the Freshwater-Concentration treatment eggs were fertilized in control water for two minutes and then transferred to test solution. After the fertilization process, the eggs were gently placed into 1-L plastic beakers filled with pre-determined TDS test solutions for the 96-hour duration of the experiment. Eggs to be used for later assays

were fertilized in freshwater, rinsed and placed in flow-through trays in vertical incubation stacks for later use.

TDS test solutions ranged in concentration from straight Salmon Creek Water (control water) or the zero added TDS concentration, to a 2500ppm TDS solution modeled after outflow from the Red Dog mine near Kotzebue, Alaska (Table 2.1). There was also a sodium chloride osmotic treatment matching the osmolality of the highest TDS solution used.

Eggs involved in the 96-hour assay were placed in approximately 1L of test solution in aerated 1L tri-pour plastic beakers maintained at ambient water temperatures (ranging from 7.0°C in October to less than 1°C in January) in a water bath. Approximately 30 eggs were used in each exposure chamber. Exact numbers of eggs/embryos were determined when the embryos reached the eyed developmental stage. Water quality measurements, consisting of DO, pH, TDS, and temperature were taken daily during the assay.

Solutions in the exposure chambers were changed after the first 48 hours. The solution was decanted to minimize disturbance to the eggs. The exposure solution was replaced by adding fresh exposure solution down the side of the exposure chamber with minimal disturbance to the embryos. Following exposure, embryos were placed in flow-through incubators and monitored for survival, physical deformity, and time to hatch. Mortalities were indicated by an opaque white coloring/appearance of the eggs and were removed as needed. Dead eggs/embryos were preserved in a 10% formalin solution in labeled Whirl Pak® bags. Preserved eggs/embryos were dissected and examined under

Table 2.1. TDS recipe used for making 2500 ppm TDS solution. Recipe models the outflow from the Red Dog Mine in Northwestern Alaska. The 2500 NaCl equiv. is the osmotic control.

2500 ppm soln	g/L	Moles	MW
CaSO4.1/2 H2O	2.3	0.0316	145.14
Na2SO4	0.14	0.0028	142.04
MgSO4	0.2	0.0032	120.37
KCl	0.02	0.0008	74.55
Total TDS added	2.52	0.0386	
2500 NaCl equiv.	1.1	0.0372	59.44

dissecting microscope. This procedure allowed us to determine the stage at which the mortality occurred, and for the detection of unfertilized eggs. Unfertilized eggs were eliminated from the experiments on developmental stages later than fertilization.

Mortality and unfertilized egg data were analyzed using analysis of variance, and regression analysis followed by Dunnett's multiple comparison procedures where appropriate. Percentage data were folded-root transformed before analysis ($Y' = \sqrt{(Y)} - \sqrt{(1 - Y)}$, Tukey 1977). Results were considered significant at $p < 0.05$.

Results

Broodyear 1999 coho salmon exposed to TDS during fertilization showed both increased overall mortalities and increased numbers of unfertilized eggs with increasing TDS concentration (Table 2.2). This result was seen in both the concentration-freshwater exposures and the concentration-concentration exposures (Figures 2.1-2.2). We looked at four separate aspects in the exposures at fertilization: short-term mortality (mortalities that occurred during the 96-hour exposure period exclusive of unfertilized eggs), long-term mortality (cumulative mortality through the button-up life stage exclusive of unfertilized eggs), unfertilized eggs, and overall percent affected (cumulative mortalities through button-up inclusive of unfertilized eggs). There was no significant effect of TDS exposure in either short-term mortalities or long-term mortalities resulting from the concentration-freshwater exposures at fertilization (Table 2.2). The concentration-concentration treatment did show a trend in long-term mortalities, though it was no

Table 2.2. Summary of 96-hour acute bioassays for BY 1999 and 2000 run during fertilization and for the subsequent 96 hours. P-values are calculated from regression analysis of the folded-root transformed data. Conc-Conc = eggs fertilized in the test solution (2 min.) and transferred to the same concentration. Conc-Freshwater = eggs fertilized in the test solution (2 min.) and transferred to control water. Freshwater-Conc = eggs fertilized in control water (2 min.) and transferred to test solution. NOEC = highest concentration that showed no significant effect. LOEC = lowest concentration which had a significant effect. Post hoc tests for both NOEC and LOEC were done using Dunnet's multiple comparisons analysis. Values for NOEC and LOEC are in ppm TDS. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up. * 2500 ppm was the highest value tested. **No mortalities at any concentration tested.

	1999 Broodyear						2000 Broodyear					
	Conc-Freshwater			Conc-Conc			Conc-Conc			Freshwater-Conc		
	P-Value	NOEC	LOEC	P-Value	NOEC	LOEC	P-Value	NOEC	LOEC	P-Value	NOEC	LOEC
Short-term Mortality	0.5190	2500*	-	0.0990	1875	2500	**	2500*	-	**	2500*	-
Long-term Mortality	0.2600	2500*	-	0.2150	2500*	-	0.9240	2500*	-	0.9510	2500*	-
% Unfertilized	0.0000	1250	1875	0.0000	1250	1875	0.0000	750	1250	0.6240	2500*	-
Overall % effected	0.0000	1250	1875	0.0000	1250	1875	0.6670	2500*	-	0.9750	2500*	-

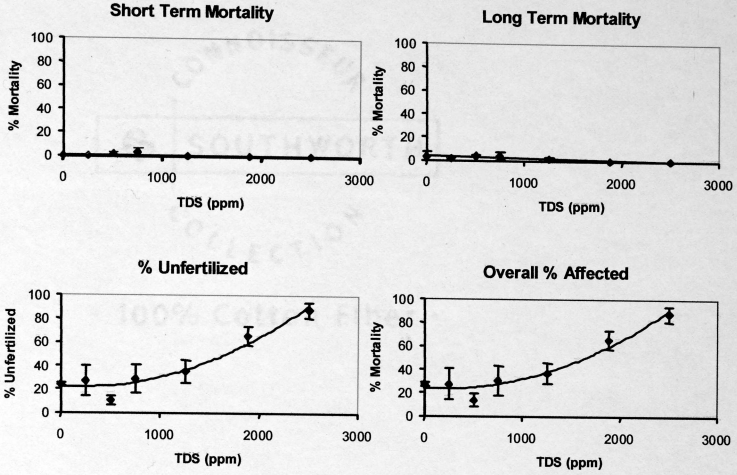

Figure 2.1. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay fertilization exposure. Eggs were fertilized in concentrations of TDS (~2 minutes) and moved to freshwater. Data show mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

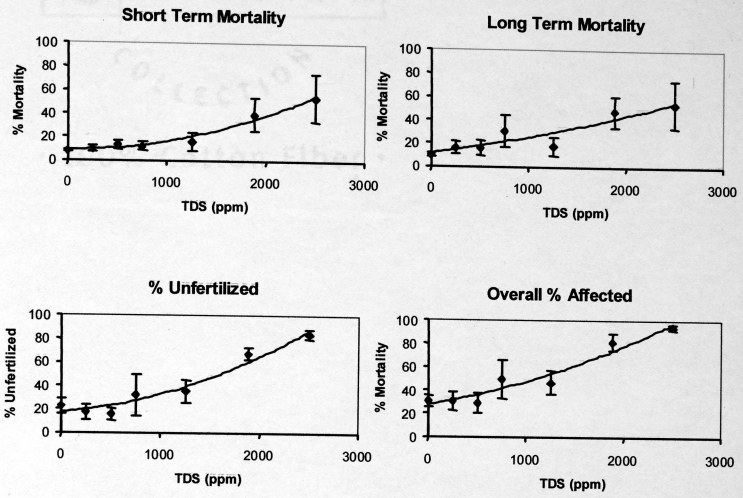

Figure 2.2. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure. Data show mean percentages of unfertilized eggs in samples $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

statistically significant. In both the concentration-concentration and concentration-freshwater exposures, there was a linear response to TDS concentration with respect to the number of unfertilized eggs and to the overall percent affected. In both cases there was a no observed effects concentration (NOEC) at 1250 ppm and a lowest observed effects concentration (LOEC) at 1875 ppm TDS. Most of the effect was from lack of fertilization (90% unfertilized at 2500 ppm TDS in both concentration-concentration and concentration-freshwater exposures). However, of those eggs in the concentration-concentration exposure that were successfully fertilized, only ~5% remained alive at the button-up life stage. Those eggs in the concentration-freshwater exposure that were successfully fertilized did survive through button-up. Mortalities of fertilized eggs were more likely to occur between the epiboly and eyed stages (Figure 2.3).

Other stages assayed during this broodyear showed no significant effects on mortality either in the short or long term (Table 2.3). The sodium chloride osmotic controls in all exposures at all life stages were not significantly different from the zero controls.

The 96-hour bioassays with broodyear 2000 coho salmon showed similar results to the previous broodyear. We again looked at short and long-term mortality, unfertilized eggs, and overall percent affected. In this broodyear, we again ran the concentration-concentration experiment, and added a freshwater-concentration exposure experiment. In the concentration-concentration exposure experiment there was once again a significant linear response of increasing numbers of unfertilized eggs with increasing concentration of TDS exposure, with an NOEC of 750 ppm and a LOEC of 1250 ppm. Again, most of

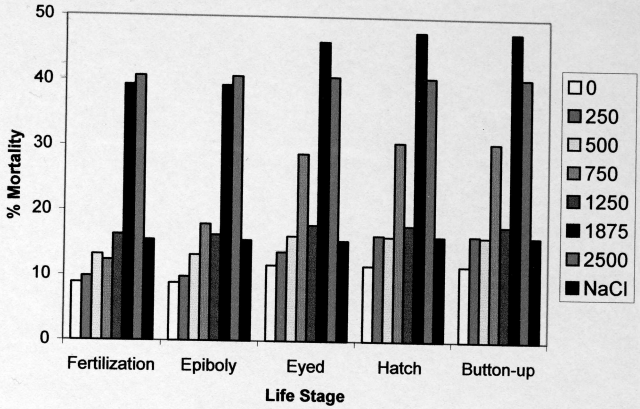

Figure 2.3. Untransformed mortality data from broodyear 1999 coho acute 96-hour bioassay at fertilization. Figure shows cumulative mortalities by concentration at specific stages of embryonic and juvenile development. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure.

Table 2.3. Summary of 96-hour acute bioassays for BY 1999 and 2000 run during stages other than fertilization. P-values are calculated from regression analysis of the folded-root transformed data. NOEC = highest concentration that showed no significant effect. Post hoc test for the NOEC was done using Dunnet's multiple comparisons analysis. Values for NOEC are in ppm TDS. Short-term mortality is mortality of fertilized eggs that occurred during the 96 hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up. * 2500 ppm was the highest value tested. **no mortalities at any concentration tested. ++ Assay was not done.

	1999 Broodyear				2000 Broodyear			
	Short-term Mortality		Long-term Mortality		Short-term Mortality		Long-term Mortality	
96-hour Bioassay Stage	P-value	NOEC	P-value	NOEC	P-value	NOEC	P-value	NOEC
BFE (Between Fertilization and Epiboly)	++	++	++	++	0.4490	2500*	0.2860	2500*
Epiboly	0.9920	2500*	0.9230	2500*	0.6060	2500*	0.5940	2500*
Eyed	**	2500*	0.5120	2500*	**	2500*	0.9440	2500*
During Hatch	**	2500*	0.7390	2500*	++	++	++	++
Post-hatch	**	2500*	0.3657	2500*	**	2500*	**	2500*
Button-up	**	2500*	**	2500*	**	2500*	**	2500*

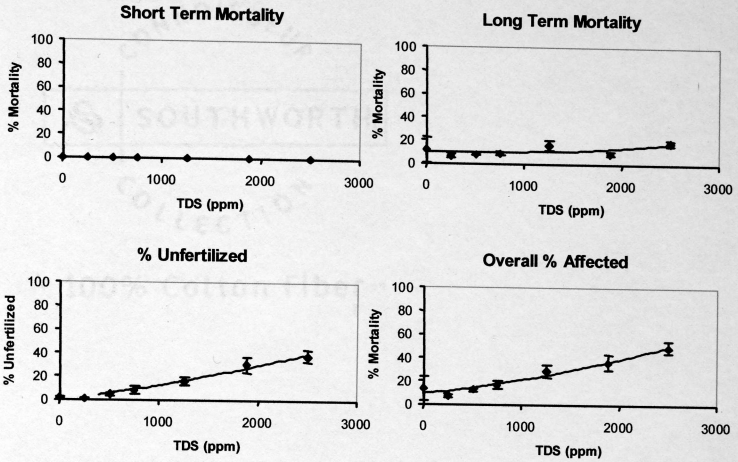

Figure 2.4. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure period. Data show mean percentages of unfertilized eggs in samples $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

the effect was from lack of fertilization (40% unfertilized at 2500 ppm). However, there were no significant trends in either short or long-term mortality (Figure 2.4, Table 2.2). The freshwater-concentration exposure produced no effect with respect to mortalities, unfertilized eggs, or overall effects (Table 2.2). Other stages assayed during this broodyear showed no significant TDS effects on mortality either in the short or long-term (Table 2.3). The sodium chloride osmotic controls in all exposures at all life stages were not significantly different from the zero controls.

Measured pH and TDS concentrations before and during exposure did not change over time. Temperature ranged at most $\pm$ 0.5° degrees. Measured pHs were in the range of 7.37 to 7.67.

Embryos/alevins were monitored following exposure to TDS through the button-up stage for obvious physical deformities such as abnormal spinal curvature. No obvious physical deformities were observed at any of the concentrations from any of the 96-hr acute bioassays.

Discussion

In salmonids as well as in several other fish species, fertilization of gametes occurs externally. Spawning fishes leave the ocean with sufficient internal food stores to sustain them as long as 12 months while at the same time providing fat stores to go into gamete maturation. Feeding ceases upon upstream migration of the adult fish. Fertilization occurs externally when eggs and spermatozoa are discharged in close proximity. Freshwater activates both eggs and spermatozoa immediately upon

immersion. Fertilization of the eggs must occur quickly because spermatozoa only remain active for 1-2 minutes following entry to the water. Eggs upon entry into freshwater will begin absorbing water and become turgid (Holliday 1969). Two things occur when these soft eggs come into contact with fresh water, water flows into the perivitelline space (between the perivitelline membrane and the chorion or outer shell) and the chorion hardens. Prior to freshwater immersion, the chorion and perivitelline membrane are indistinguishable from one-another except upon microscopic inspection. The process of water entrance as well as hardening of the chorion has been shown to prevent the subsequent fertilization of trout eggs (Hoar 1957). However, the uptake of water and hardening of the chorion can be varied experimentally. Potts and Rudy (1969) in their work with Atlantic salmon theorized that there are changes in the permeability of an egg to water and other ions following release into the environment, and that specific ions such as calcium may delay the initial stages of high permeability leading to water hardening of an egg. They found that permeability decreases most quickly in distilled water and that while the onset of this stage of high permeability may be delayed by the presence of calcium (10 mM/L), it may be delayed indefinitely in high sodium concentrations (145 mM/L) causing these embryos to never fully water harden.

Fertilization of the salmonid embryo causes a chain of chemical events to occur beginning with fusion of the sperm to the egg and culminating in the release of cortical alveoli causing a wave of calcium within the egg from the animal pole to the vegetal pole (Hart, 1990, Smith 1967). Information is conflicting as to whether calcium is taken up or given off during the fertilization event. Khlebovich *et al.* (1977) in a study of

perivitelline fluids found that during the event of fertilization the eggs release sodium ions into the environment and take up calcium ions from the environment. Hayes *et al.* (1946) found that salmon eggs release sodium, calcium and chloride ions. Movement of these ions appeared to cease between six and twelve hours. Fertilization is thought to require the presence of small amounts of calcium or magnesium ions in the water (Blaxter 1969). However, eggs will fertilize in distilled water (Smith 1967).

Salmonid exposure to high levels of TDS has shown mixed results. Ketola *et al.* (1988) found that exposing salmonid embryos to high concentrations of calcium (520 mg/L or greater) during water hardening decreased the survival rates of several salmonid species. Ketola *et al.'s* methodology differed from ours in that the embryos used in that study were dry fertilized (fertilized in the presence of ovarian and seminal fluids only), while embryos in this study were fertilized in control or exposure waters. Both studies rinsed fertilized embryos in exposure waters. Embryos in the Ketola *et al.* study were water-hardened (1.5-3 hour exposure) in exposure solutions then grown out through eye-up in incubation stacks. Ketola *et al.'s* study most closely resembles our broodyear 2000 freshwater-concentration 96-hour acute bioassay. Our results differed from theirs in that they found survival to eye-up to be significantly reduced in calcium concentrations of 520 mg/L and above, while we found no significant trends in mortality. Both our study and theirs investigate increased TDS levels though Ketola *et al.* examine the water chemistry of natural systems and ours explores a simulated industrial effluent. A comparison of results of the two shows them to be inconsistent. Chapman *et al.* (2000) exposed both chironomid (*Chironomus tentans*) larvae and embryonic and juvenile

rainbow trout (*Oncorhynchus mykiss*) to two synthetic TDS mixtures modeled after the ionic composition of two mine effluents from Alaskan mining operations. Both life stages of rainbow trout, and the chironomid larvae were exposed to concentrations of TDS up to 2000 mg/L. No significant effects of the exposures were found on the rainbow trout. However, significant effects appeared in the chironomid larvae above 1100 mg/L. Our coho embryos exposed at these (BY 2000 freshwater-concentration at fertilization and BY 1999 & 2000 button-up fry) life stages also showed no significant trend in mortalities. We found these life stages, and several others to be unaffected by TDS exposure in either the short or long term. However, when the coho were exposed at fertilization, significant effects were observed. We found coho salmon to be sensitive to TDS exposure at fertilization but not at other embryonic life stages or the juvenile stages from alevin to button-up.

It is important to look at not only the immediate effects of exposure, but to look also at later or delayed effects of exposure to a toxicant. Eggs in our experiment that were fertilized had more problems later on. Eggs exposed at fertilization tended to survive to the eyed stage, but those exposed to the higher concentrations (1875 and 2500 ppm TDS) had fairly high mortality rates between the eyed and alevin stages (figure 2.3). In the 2500-ppm concentration we had 50 percent mortality of the 50 percent that were fertilized in broodyear 1999.

A study done in conjunction with this research looked at the individual ionic components of our TDS mixture and the impact of those ions on the fertilization rates of king and pink salmon (Brannok *et al.* 2002). These ions were tested individually at levels

equivalent to our 2500ppm simulation, also at one quarter of the concentration and at four times the concentration. Fertilization rates in both the king and pink salmon were significantly lower with exposure to either calcium or sulfate at 2500ppm equivalent. Potassium and magnesium ions showed no detectable differences from the control at that level. This work pointed to calcium or possibly the sulfate as being the likely cause of lowered fertilization rates in our experiments. Mount *et al.'s* (1997) work on individual ions and recognized test species targeted potassium as the major factor impeding survival. In Brannok *et al.*'s (2002) work potassium was found not to be a factor in the fertilization rates of salmonids at any of the levels of exposure.

Based on our results, it may be prudent to reexamine the TDS discharge limitations as they are now specified by regulation. TDS is measured in parts-per-million (ppm) or milligrams-per-liter (mg/L). But, this measurement tells nothing of the chemical components, concentrations of individual components, or interactions of any of those chemicals. The difficulty in setting TDS limits comes from the wide variation of the individual components of TDS mixtures. Several of these chemicals occur in natural systems through surface runoff or groundwater leaching. Natural systems can range anywhere from 20 ppm TDS to well over 100,000 ppm TDS (Baulding 1976, Dickerson & Vinyard 1999, Gosselin 1997). The toxicity of TDS may be either as an osmotic effect, the result of a single ionic species or a combination of two or more ions. Our NaCl osmotic control has eliminated an osmotic effect. In our experiments the likely cause of unsuccessful fertilization is likely to be calcium ions.

Conclusions

TDS toxicity is dependent upon both the composition and concentration of individual ions. Toxicity can't be assessed simply on the basis of a TDS measurement in mg/L. While TDS occurs naturally in watershed systems, it is the anthropogenic sources of TDS that are cause for concern. More research needs to be done before limits on TDS are set regarding discharge into natural waters in Alaska. Site-specific assays can be used to assess toxicity with regards to TDS (Chapter 3). One possibility for research would be to compare two or several different stocks of the same species, one wild and one a cultured stock, or several stocks from different water sources and see what differences arise. Individual ions and compounds commonly found in discharges should be tested with a salmonid species found both locally, and throughout the state of Alaska or for wherever the standards are being set.

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Chapter 3

Methods for Conducting Short-term Toxicity Tests with Salmonid Eggs at Fertilization[2]

[2] Failor-Rounds, B.J. 2003. Methods for Conducting Short-term Toxicity Tests with Salmonid Eggs at Fertilization. Prepared for submission in Transactions of the American Fisheries Society.

Abstract

We have developed a method for performing short-term (24-hour) embryo toxicity studies during salmonid egg fertilization. The specific assay developed exposed unfertilized and fertilized salmonid eggs to various concentrations of total dissolved solids (TDS) in fresh water. Exposure to the test solutions occurred in one of three methods: during only the first two minutes of fertilization, two minutes after addition of sperm, continuously from the addition of sperm to the end of the assay. The endpoint for the assay is the success of egg fertilization, which is easily identifiable at the 4 to 8 cell stage of the embryo. Six species of salmonids were tested using the developed assay. Control embryo fertilization rates averaged 95% across the six species, whereas in the highest concentration of TDS tested the average fertilization success was less than 10%. Minimum discernable statistically significant differences from the control were 10%. Results suggest that the assay can be generalized across the species and may be useful in setting site-specific criteria for discharging wastes.

Introduction

Several short-term bioassay methodologies are available to assess the impacts of toxicants on receiving waters and their inhabitants. Often the early life stages of an organism are the most sensitive to effects of pollutants. Embryonic life stages of saltwater organisms such as bivalve (Ramachandran *et al.* 1997, Ringwood 1992), crab (Ramachandran *et al.* 1997), kelp (Kevekordes 2002), polychaete (Hutchinson *et al.* 1995), sea urchin (Böttger & McClintock 2001, Krause 1994, Ramachandran *et al.* 1997, Ringwood 1992), and shrimp (Kline & Stekoll 2000) species have been the subject of several toxicology studies. Toxicology studies involving the embryonic life stages of the freshwater invertebrates (Chapman *et al.* 2000) and zebrafish (Samson & Shenker 2000) are plentiful as well. Salmonids have been widely used as the subject of toxicology studies as juveniles (Chapman *et al.* 2000, Wan *et al.* 1992, Zitko & Carson 1976), but less frequently as embryos (Canaria *et al.* 1999, Chapman *et al.* 2000, Ketola *et al.* 1987, Ketola *et al.* 1988). No published studies have assessed the effects of toxicants on salmonid fertilization.

Standardized assays available to test industrial effluents and their impacts on a general receiving system (Duodoroff *et al.* 1951, U.S. EPA 1991) may not be applicable to indigenous species of an area. In order to write site-specific criteria regarding discharge of industrial effluents to receiving waters, applicable assays must be run using the site-specific effluent or a precise synthetic mock-up of that effluent and a site-specific species. LeBlond and Duffy (2001) found that using Microtox® and *Selenastrum*

capricornatum assays and a non-site-specific synthetic TDS mixtures is a poor predictor of the toxic effects of a specific discharged effluent to a receiving system.

This paper describes methods for conducting a short-term (~ 24 hour) embryo toxicity test with salmonids incorporating fertilization and water hardening. The described methods not only incorporate the two developmental stages but also attempt to distinguish which stage is more sensitive. The bioassay was run comparing the response of six species of salmonids to a synthetic effluent that simulates outflows from the Red Dog Mine in northwestern Alaska.

Methods

The experimental animals of this project were coho (*Oncorhynchus kisutch*), chum (*Oncorhynchus keta*), king (*Oncorhynchus tschawytscha*), pink (*Oncorhynchus gorbuscha*), steelhead (*Oncorhynchus mykiss*) salmon, and arctic char (*Salvelinus alpinus*). Broodstock for the coho, chum, king, and pink salmon were obtained as mature fish returning to Douglas Island Pink and Chum Inc.'s (DIPAC) Macaulay Hatchery located in Juneau, Alaska. Gametes for the steelhead fertilization trial were taken at the NOAA Little Port Walter hatchery facility, Baranof Island, Alaska by hatchery staff and transported to Juneau via floatplane. Gametes for the arctic char fertilization trial were taken from captive broodstock residing in the university wet-lab area at DIPAC's Macaulay Hatchery. Bioassays took place in the university wet lab area in the Macaulay Hatchery.

For each bioassay three female and three male salmon of a single species were spawned. The bellies of the fish were wiped down with an antiseptic iodine solution. Females were spawned (Piper *et al.* 1986) into separate 1-gallon Ziploc® bags. The bags were then sealed and placed into a cooler for transportation to the wet-lab area. Males were spawned (Piper *et al.* 1986) separately into Dixie® cups to monitor quality. Samples with excessive blood or feces in the milt were discarded and another male was used. The Dixie cups were then placed in separate 1-quart Ziploc bags and also placed in the cooler for transportation to the wet-lab area. In the wet-lab, the eggs were pooled in a disinfected plastic bowl, and the milt was pooled in a disinfected 200-mL plastic cup. Eggs were fertilized in one solution and either moved to a container of that same solution for the duration of the experiment, or moved to another solution following rinsing of the eggs (see below). This procedure resulted in the three possible combinations of exposure: concentration-concentration (fertilized in TDS solution and moved to same TDS, labeled C-C), concentration-freshwater (fertilized in TDS and moved to control water, labeled C-F), freshwater-concentration (fertilized in control water and moved to TDS solution, labeled F-C). In addition the control consisted of fertilization in control water and then moved to control water. TDS solutions ranged in concentration from straight Salmon Creek Water (control water) or the zero added TDS concentration, to a 2500ppm TDS solution modeled after the outflow from the Red Dog mine near Kotzebue, Alaska (Table 3.1). There was also a sodium chloride osmotic treatment matching the osmolality of the highest TDS solution used.

Table 3.1. TDS recipe used for making 2500 ppm TDS solution. Recipe models outflows from the Red Dog Mine in Northwestern Alaska. The 2500 NaCl equiv. is the osmotic control.

2500 ppm soln	g/L	Moles	MW
CaSO4.1/2 H2O	2.3	0.0316	145.14
Na2SO4	0.14	0.0028	142.04
MgSO4	0.2	0.0032	120.37
KCl	0.02	0.0008	74.55
Total TDS added	2.52	0.0386	
2500 NaCl equiv.	1.1	0.0372	59.44

The arctic char spawning differed a little from the above methodology in that the adult fishes were not sacrificed. Mature adults were anesthetized in MS222 (methane-tricaine-sulfonate) and spawned by the live fish method described in Piper *et al.* (1986). From that point the methodology continued as described above.

Approximately 75-100 eggs were placed in a cup containing 100 mL of the fertilization solution. Milt (0.2 mL) was added immediately to the cup with a syringe. The cup was filled (another 100 mL) with the solution to facilitate mixing of the milt and eggs. Eggs were then allowed to sit for two minutes followed by rinses with the exposure solution until the water poured off was clear (usually two rinses). Fertilized eggs were gently placed into 1-L plastic containers filled with the pre-determined TDS solutions for the first 24 hours. Each beaker was provided with an aeration device and placed in a water bath for temperature control. Conductivity and temperature were recorded for all treatments.

All species tested were exposed from fertilization until they reached the 4-8-cell stage (approximately 24 hours based on time/temperature dependent development rates). At the end of the exposure the fertilized eggs were strained out of the exposure solution and placed in labeled Whirl Pak® bags. Stockard's solution (Velsen 1980) was added to the bags to preserve the eggs and facilitate clearing of the eggshells. The eggs were then examined under a dissecting microscope to determine the cellular stage of the egg. If the cell mass was not clearly visible through the shell, eggs were dissected to determine whether cleavage had occurred. If an egg lacked visible cleavage, it was considered to be unfertilized.

Mortality and unfertilized egg data were analyzed using analysis of variance, and regression analysis, followed by Dunnett's multiple comparison procedures to determine NOEC (No-observed-effects concentration) and LOEC (lowest-observed-effects concentration) values. Percentage data were folded-root transformed prior to analysis ($Y' = \sqrt{(Y)} - \sqrt{(1 - Y)}$, Tukey 1977). Results were considered significant at $p < 0.05$.

Results

In the concentration-concentration exposures all species tested showed a significant trend of increasing numbers of unfertilized eggs with increasing TDS concentration exposure (Figures 3.1-3.6, Table 3.2). The LOEC varied among species. Chum and steelhead had an LOEC of 750 ppm while the king, pink, and coho salmon exhibited a lower LOEC of 250 ppm TDS. Arctic char appear to be the most tolerant of TDS exposure in this experiment because the lowest concentration that had a significant effect was 1875 ppm. In this series of experiments the smallest detectable difference was about 10%.

In the concentration-freshwater experiment, chum, steelhead, king, pink and coho salmon showed increased numbers of unfertilized eggs with increasing TDS concentration exposures (Figures 3.1-3.6, Table 3.2). The char were not affected even at the 2500ppm level. This experiment however, did not show any groupings of species by response as observed in the concentration-concentration exposure. Every species exhibited a different LOEC ranging from coho salmon at 250 ppm TDS, to chum salmon at 1875 ppm TDS (nearly the highest concentration tested).

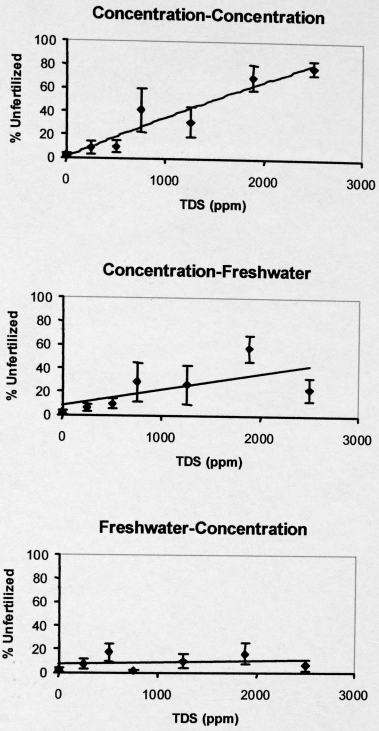

Figure 3.1. Untransformed data from broodyear 2000 chum salmon 24-hour fertilization trial (07/23/00-07/24/00, 8.5°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

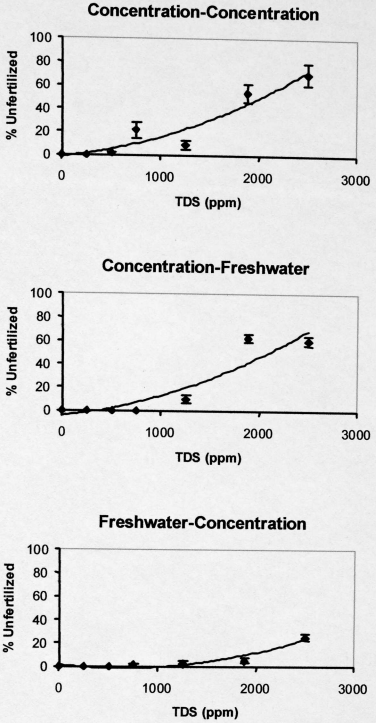

Figure 3.2. Untransformed data from Broodyear 2001 steelhead salmon 24-hour fertilization trial (05/25/01-05/26/01, 6.2°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

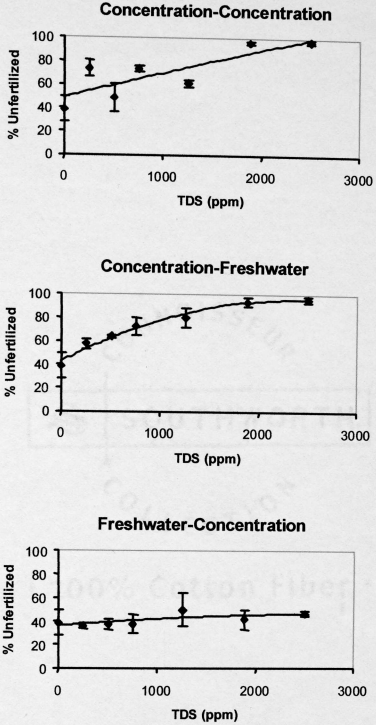

Figure 3.3. Untransformed data from broodyear 2001 king salmon 24-hour fertilization trial (08/03/01-08/04/01, 7.9°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

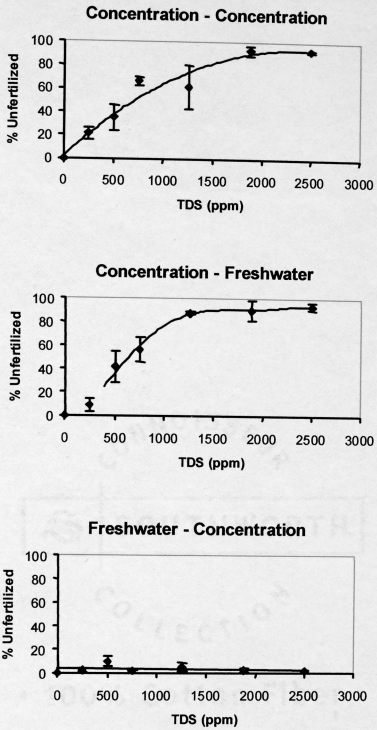

Figure 3.4. Untransformed data from broodyear 2001 pink salmon 24-hour fertilization trial (08/13/01-08/14/01, 8.1°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

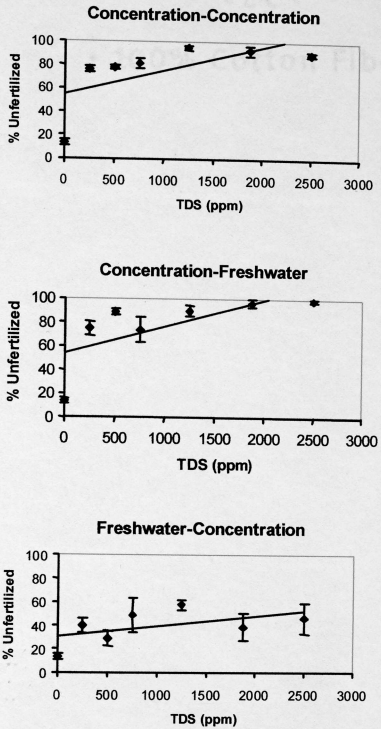

Figure 3.5. Untransformed data from broodyear 2001 coho salmon 24-hour fertilization trial (10/24/01-10/25/01, 6.3°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

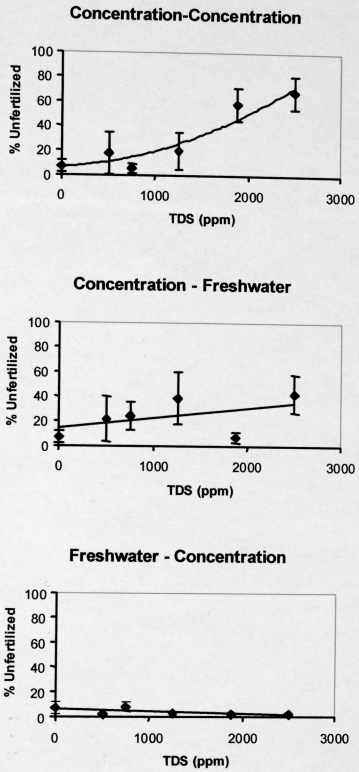

Figure 3.6. Untransformed data from broodyear 2001 arctic char 24-hour fertilization trial (11/10/01-11/12/01, 4.5°). Data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

Table 3.2. Results of the three types of 24-hour fertilization trials for 6 species of fish. P-values are calculated from regressions on folded-root transformed data. Conc-Conc = eggs fertilized in the test solution (2 min.) and transferred to the same concentration. Conc-Freshwater = eggs fertilized in the test solution (2 min.) and transferred to control water. Freshwater-Conc = eggs fertilized in control water(2 min.) and transferred to test solution. NOEC = highest concentration that showed no significant effect. LOEC = lowest concentration which had a significant effect. Post hoc tests for both NOEC and LOEC were done using Dunnet's multiple comparisons analysis. Values for NOEC and LOEC are in ppm TDS. * 2500 ppm was the highest value tested. **At least 1 concentration higher not significantly different from control.

	Conc-Conc Exposure			Conc-Freshwater Exposure			Freshwater-Conc Exposure		
	P-value	NOEC	LOEC	P-value	NOEC	LOEC	P-value	NOEC	LOEC
Chum Salmon *(Oncorhynchus keta)*	0.0000	500	750**	0.0270	1250	1875**	0.4010	2500*	-
Steelhead Salmon *(Oncorhynchus mykiss)*	0.0000	500	750**	0.0000	750	1250	0.0000	1250	1875
King Salmon *(Oncorhynchus tschawytscha)*	0.0000	0	250**	0.0000	500	750	0.8690	2500*	-
Pink Salmon *(Oncorhynchus gorbuscha)*	0.0000	0	250	0.0000	250	500	0.1640	2500*	-
Coho Salmon *(Oncorhynchus kisutch)*	0.0001	0	250	0.0000	0	250	0.0820	500	750**
Arctic Char *(Salvelinus alpinus.)*	0.0060	1250	1875	0.4090	2500*	-	0.4330	2500*	-

In the freshwater-concentration exposure, the only species to show a significant effect was the steelhead salmon. For this experiment mortalities were significantly correlated with TDS concentration. The LOEC for steelhead was 1875 ppm (Figure 3.2, Table 3.2). All other species tested showed no effect of TDS on the number of unfertilized eggs. Although coho salmon fertilization was significantly reduced at 750ppm, fertilizaton success was not affected at the higher concentrations tested (Figure 3.5, Table 3.2).

The response of eggs from all species to the sodium chloride osmotic control was never significantly different from the zero control in any of the exposures (Figures 3.1-3.6).

Temperature, pH, and TDS concentrations were measured prior to the beginning of each set of experiments and, with the exception of temperature, did not change over time. Since developmental rates of salmonids are temperature dependent, temperature was measured before, during and at the end of the exposure time in order to calculate an average temperature by which to predict development (Groot & Margolis 1998). The water temperatures varied (4.5-8.5°C) among the experiments since these species of salmonids spawn at different times of the year, and we were using water at the ambient temperature (Table 3.3).

We were interested in exposing the eggs/embryos for the time from fertilization to the 4-8-cell stage because this time frame not only made it easier to read the fertilization success, but also ensured that the species were all exposed for approximately the same

Table 3.3. Summary of dates, average water temperatures during exposure and exposure duration by species of salmonid used in fertilization exposures.

Species	Dates	Water Temperature (C)	Exposure Duration
Chum Salmon *(Oncorhynchus keta)*	07-23-00 to 07-24-00	8.5	21 hrs
Steelhead Salmon *(Oncorhynchus mykiss)*	05-25-01 to 05-26-01	6.2	23 hrs
King Salmon *(Oncorhynchus tschawytscha)*	08-03-01 to 08-04-01	8.8	19 hrs
Pink Salmon *(Oncorhynchus gorbuscha)*	08-13-01 to 08-14-01	9	18 hrs
Coho Salmon *(Oncorhynchus kisutch)*	10-24-01 to 10-25-01	6.3	19 hrs
Arctic Char *(Salvelinus alpinus)*	11-10-01 to 11-12-01	4.5	43 hrs

developmental period. The time of exposure varied from 18 hours for pink salmon to 43 hours for arctic char (Table 3.3).

Discussion

Only a few studies have reported bioassays with salmonid embryos. Canaria *et al.* (1999) developed a 7-day salmonid embryo bioassay exposing embryos to either pulp or paper mill effluents or a reference toxicant (sodium dodecyl sulphate), with an easily readable endpoint of the epiboly stage of development. This stage can be detected by the presence of a visible white line in the embryo. Embryos in this test were dry fertilized (fertilized not in the presence of any solution exclusive of ovarian or seminal fluids) prior to exposure to the test solution. Embryos that did not reach the epiboly developmental stage were considered unsuccessful. The endpoint and measure of successful or unsuccessful development of embryos in their assay may be due to either exposure to the toxicant or to the fact that the egg was never fertilized. Differentiation of the two possibilities in this case is problematic because, though an embryo may form an initial mass at the animal pole, if that egg is unfertilized, the mass will begin to degenerate. That same thing happens to a fertilized egg that experiences mortality very early in development. Therefore both embryos that were initially unfertilized and embryos that were fertilized and died soon after will appear the same visually. Ketola *et al.* (1987, 1988) exposed newly fertilized trout and salmon eggs to different concentrations of hard water solutions to examine impacts of this exposure on water hardening of the embryos. Embryos were fertilized in the presence of ovarian fluid, rinsed with exposure waters,

and water-hardened in exposure solutions for 1.5 to 3 hours following which the embryos were incubated through the eyed stage of development. Three species of salmonids were investigated: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*). After exposure the fish were allowed to grow out in order to determine survival rates and the effect of exposure during water hardening on the survival rates of these three species. The similar exposure in our study would be the freshwater-concentration study. With coho salmon, we found an LOEC of 750, while Ketola *et al.* (1988) observed significant effects on survival to the eyed stage using calcium in excess of 520 mg/L. Chapter 2 of this thesis addresses the issue of grow-out post exposure of embryos. The broodyear 2000 freshwater-concentration 96-hour bioassay at fertilization most closely relates to Ketola *et al.* (1988) exposing embryos and continuing to monitor survival for an extended time. The study conducted in chapter 2 found no differences results when embryos were water-hardened in either the control water or exposure solutions.

Salmon spawning in impacted systems will expose the gametes from the time they are expelled even prior to the fertilization event. The methodology used in this study specifically assessed the fertilization rates of fish as a function of the test solutions. Using this methodology the eggs are fertilized in the test solution, closer to what would happen in a natural system. Eggs were fertilized in either the TDS solution or the control water and were then moved to either the same TDS solution or to control water. Thus, the experimental is one of reciprocal exposures: control-to-control (F-F), TDS to TDS (C-C), control to TDS (F-C) and TDS to control (C-F). The actual measure of effect was

to determine which eggs had reached the easily visible 4-8-cell stage of development. Using this complete combination of possible crosses in the exposure solutions we were able to determine whether the TDS had its effect at fertilization or during water hardening and we could determine which of the two developmental stages was more sensitive to the pollutant.

We found differing fertilization rates among the six species of salmonids tested when exposed only to the control water. It is common for different species to exhibit differing fertilization rates (Groot & Margolis 1998). In addition, there are several possible sources of fertilization variability including environmental factors and parental fitness. Some of the species (chum, steelhead, and pinks) we used exhibited 100% fertilization rates in the control water. Of those that did not, it is likely that the fish were not completely ripe. When spawning the king and coho salmon for example, we noted that there was a high incidence of "green" eggs (eggs still in their skein). Fertilization rates of coho salmon observed in those fishes used in the study discussed in chapter 2 ranged from 80-100% fertilized embryos.

Even though some of the fertilization rates in the controls were different, the slopes of the regressions of percent fertility versus TDS concentration were not significantly different among the different species. This result suggests that, although fertilization rates in freshwater varied, the embryos response to the toxicant was similar. That is, the same concentration of solution evoked the same percent response difference from the control solution. Therefore, we believe this assay is valid even for fish with lower fertilization success. However, in order to obtain more statistical power we

recommend that eggs that do not flow freely from the female should not be used since they are usually not sufficiently ripe for this type of assay (Piper *et al.* 1986).

Conclusions

Our results suggest that this assay can be generalized for use on any species within the salmonid fishes. It is not known whether this assay can be generalized with respect to other toxicants or other fish orders. For that to be known, further testing is needed using other model toxicant and other species. The assay reported here is a relatively easy and cheap short-term bioassay that yields results quickly and requires minimal training. The assay does not, however, yield any information about the possibility of long-term or delayed effects. For such responses to be monitored, a grow-out period is needed in addition to the above-described methodology. The study related in chapter 2 found significant effects on the long-term survival of embryos exposed at fertilization to our model toxicant. Embryos that survived exposure at fertilization to the highest exposure concentration experienced 100% mortality by the button-up life stage. So, while the methodology described here will yield results quickly, there are other aspects of exposure to a toxicant that will not be seen.

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Appendices

Appendix A

Figures of untransformed and folded root transformed data from the 1999 and 2000 broodyear 96-hour acute salmonid bioassays with coho salmon embryos and juveniles.

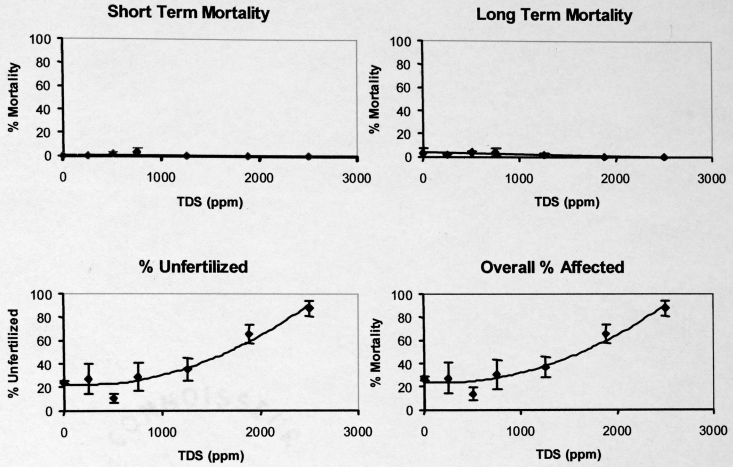


Figure A.1. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay fertilization exposure. Eggs were fertilized in concentrations of TDS (~2 minutes) and moved to freshwater. Data show mean percentages of unfertilized eggs in samples $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

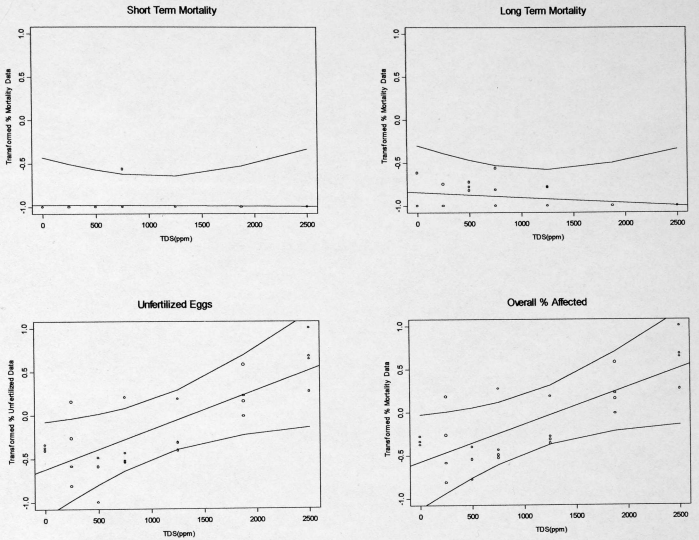


Figure A.2. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay fertilization exposure. Eggs were fertilized in concentrations of TDS (~2 minutes) and moved to freshwater. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

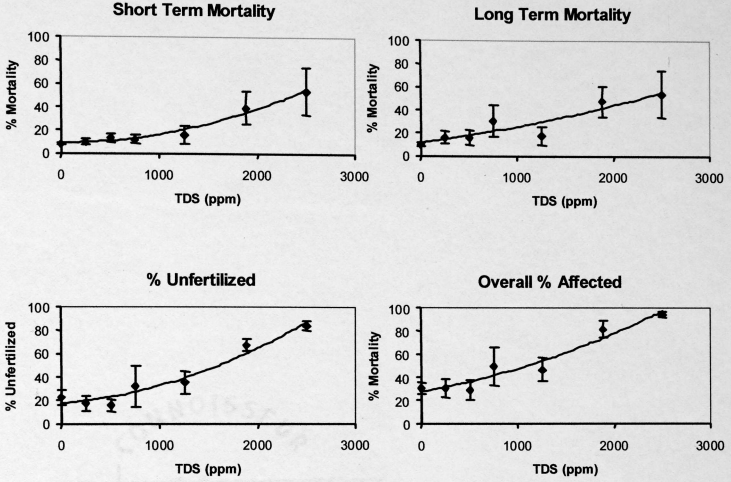


Figure A.3. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure. Data show mean percentages of unfertilized eggs in samples $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

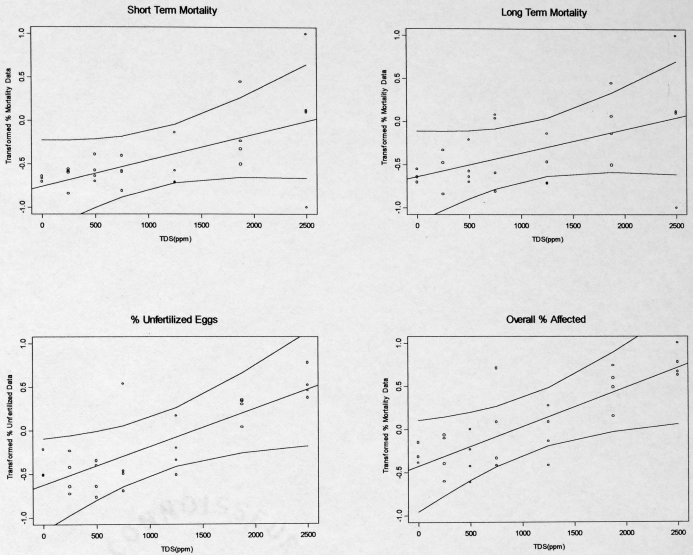


Figure A.4. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

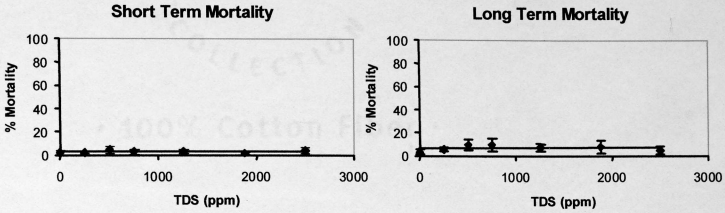


Figure A.5. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the epiboly life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

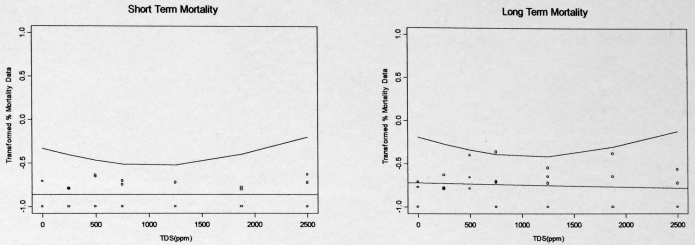


Figure A.6. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the epiboly life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

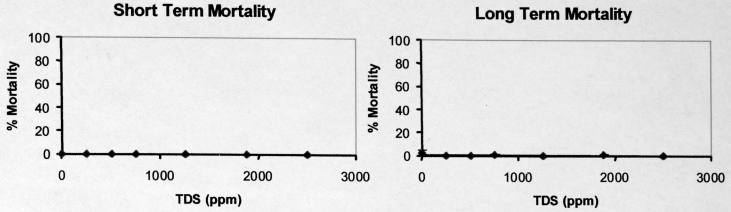


Figure A.7. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the eyed life stage. Plots show mean mortality percentages $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

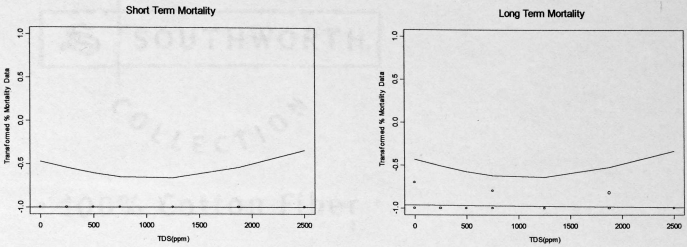


Figure A.8. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the eyed life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

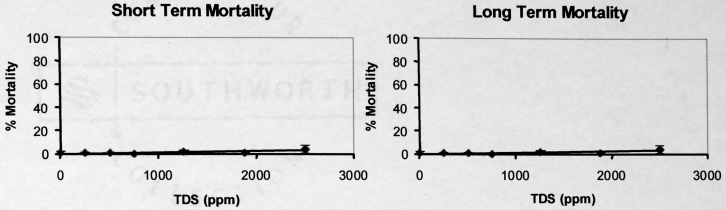


Figure A.9. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the pre-hatch life stage. Plots show mean mortality percentages $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

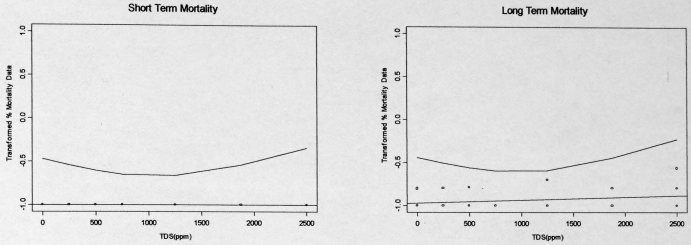


Figure A.10. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the pre-hatch life stage. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

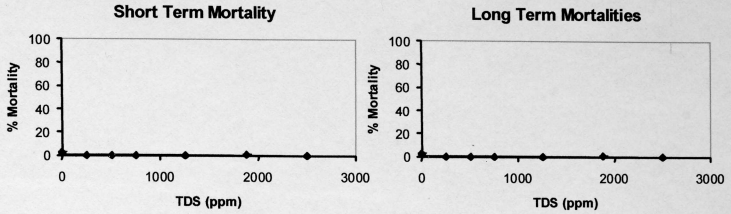


Figure A.11. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the post-hatch life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

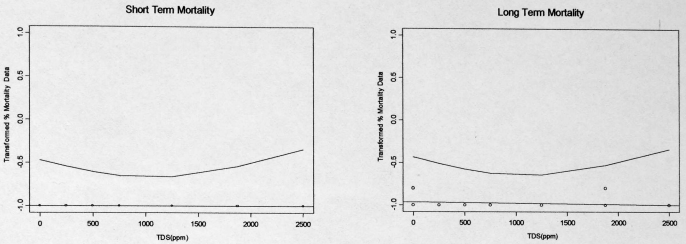


Figure A.12. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the post-hatch life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

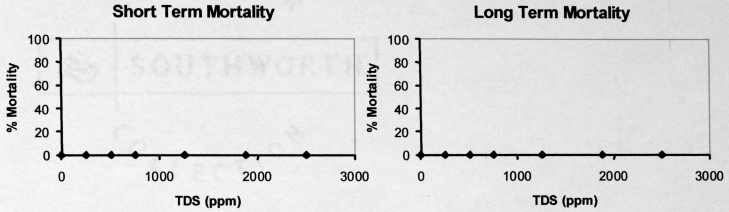


Figure A.13. Untransformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the button-up life stage. Plots show mean mortality percentages $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

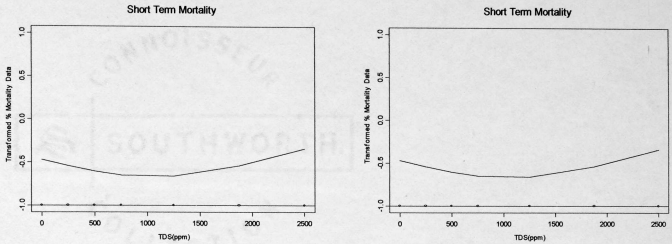


Figure A.14. Folded root transformed data from broodyear 1999 coho salmon 96-hour acute bioassay at the button-up life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

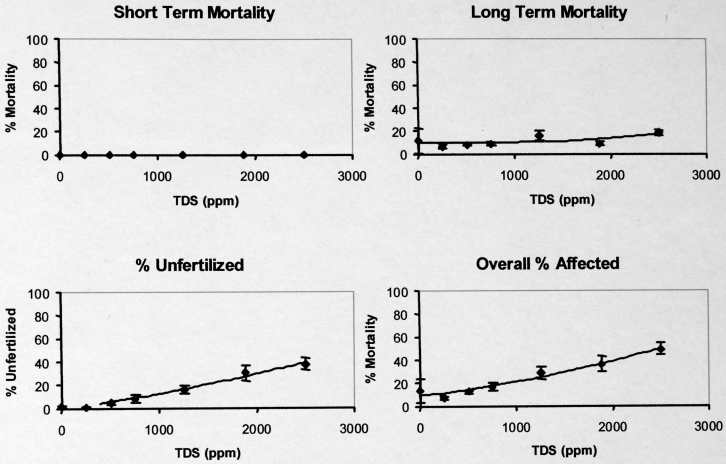


Figure A.15. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure period. Data show mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

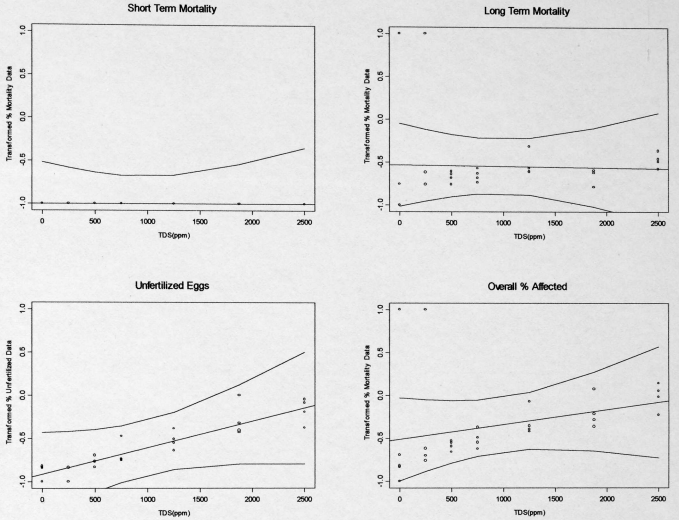


Figure A.16. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at fertilization. Eggs were fertilized in and remained in concentrations of TDS. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

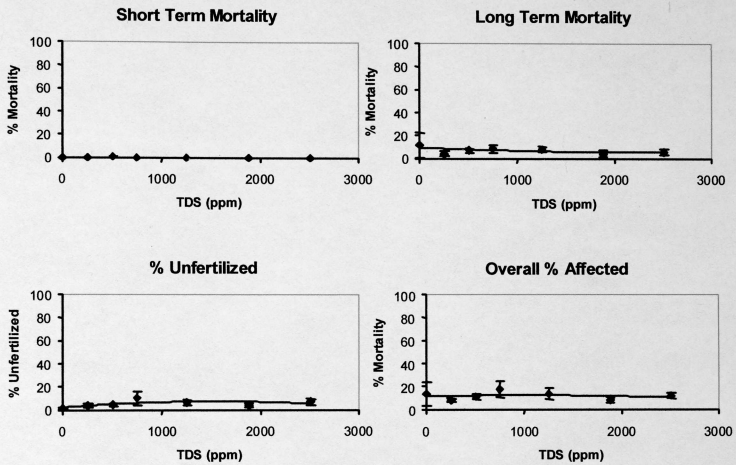


Figure A.17. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay freshwater fertilization exposure. Eggs were fertilized in freshwater (~2 minutes) and moved to concentrations of TDS for the 96-hour exposure period. Data show mean percentages of unfertilized eggs in samples $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

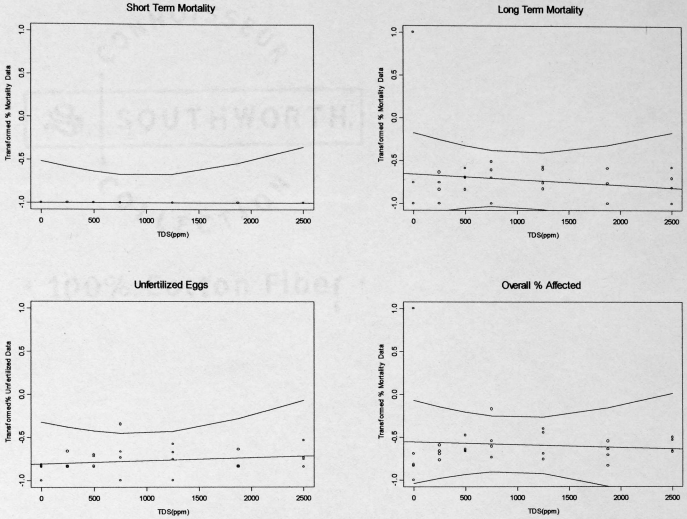


Figure A.18. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay freshwater fertilization exposure. Eggs were fertilized in freshwater (~2 minutes) and moved to concentrations of TDS for the exposure period. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period exclusive of unfertilized eggs. Long-term mortality is the cumulative mortality occurring between fertilization and button-up also exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

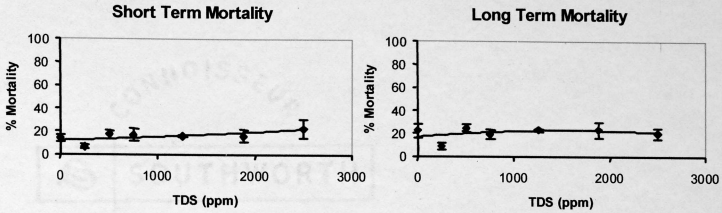


Figure A.19. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the BFE (between fertilization and epiboly) life stage. Plots show mean mortality percentages $\pm$ standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

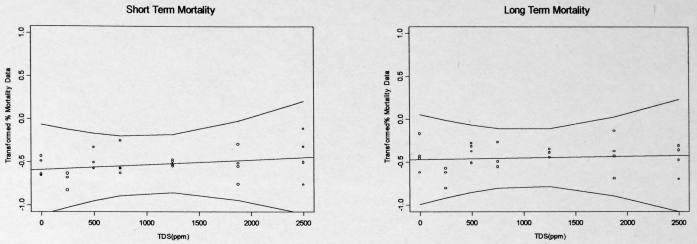


Figure A.20. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the BFE (between fertilization and epiboly) life stage. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

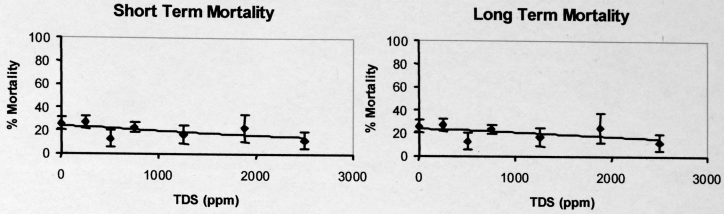


Figure A.21. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the epiboly life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

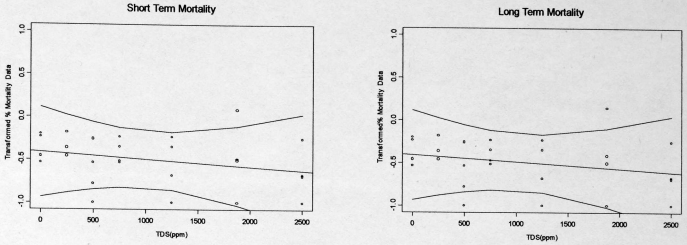


Figure A.22. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the epiboly life stage. Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

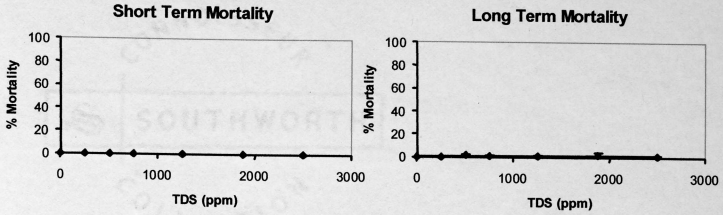


Figure A.23. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the eyed life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

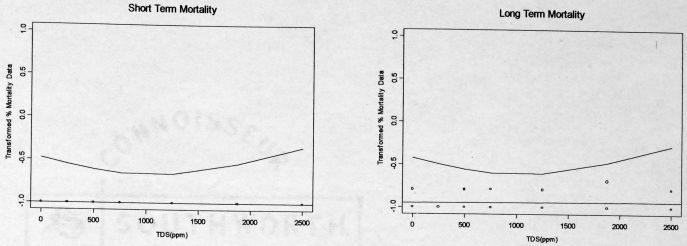


Figure A.24. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the eyed life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

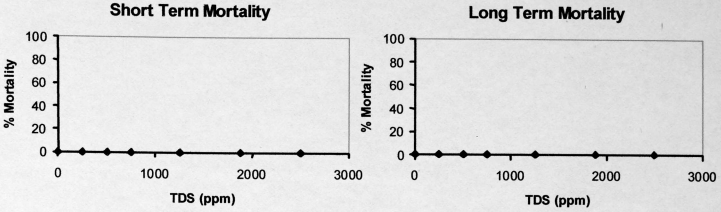


Figure A.25. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the post-hatch life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

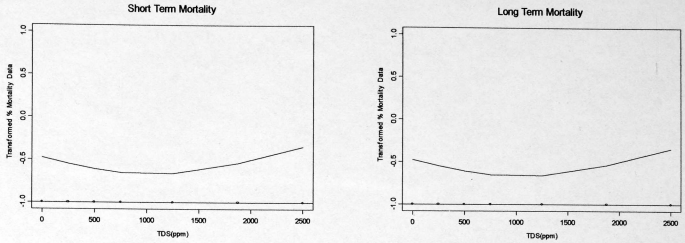


Figure A.26. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the post-hatch life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

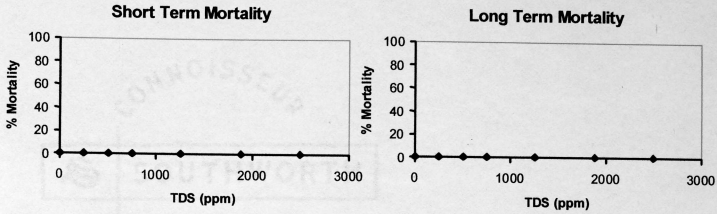


Figure A.27. Untransformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the button-up life stage. Plots show mean mortality percentages ± standard error bars and a trend line. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

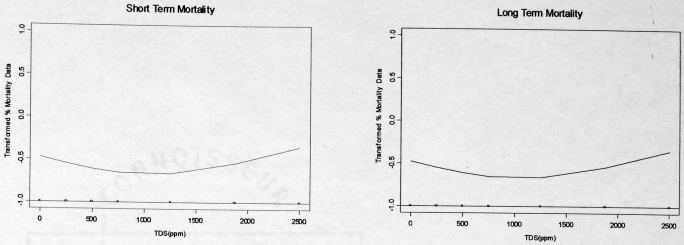


Figure A.28. Folded root transformed data from broodyear 2000 coho salmon 96-hour acute bioassay at the button-up life stage. Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Short-term mortality is mortality that occurred during the 96-hour exposure period. Long-term mortality is the cumulative mortality occurring between exposure and button-up.

Appendix B

Figures of folded root transformed data from the 24-hour fertilization bioassays utilizing salmonid embryos.

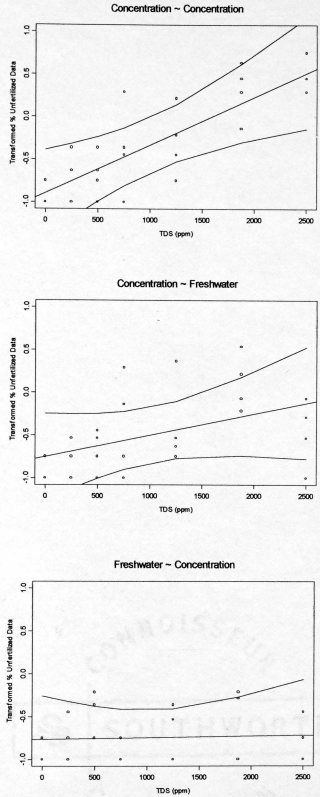
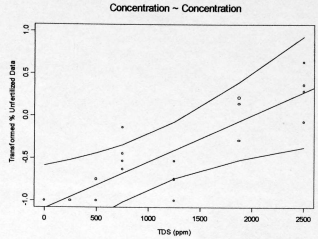
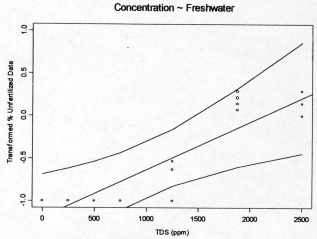


Figure B-1. Folded root transformed data from broodyear 2000 chum salmon 24-hour fertilization trial (07/23/00-07/24/00, 8.5°). Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.





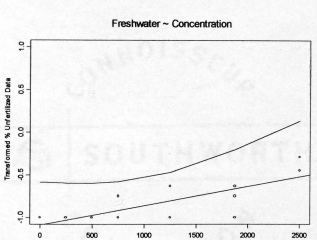


Figure B-2. Folded root transformed data from broodyear 2001 steelhead salmon 24-hour fertilization trial (05/25/01-05/26/01, 6.2°). Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

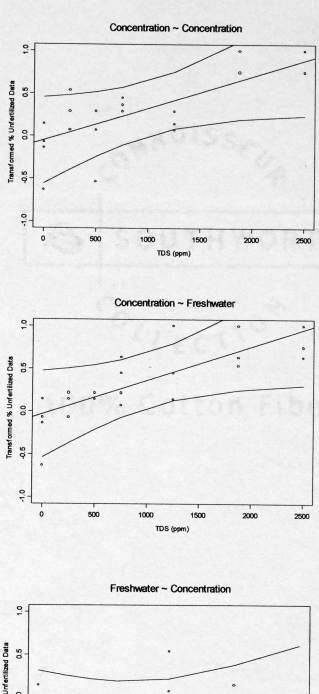
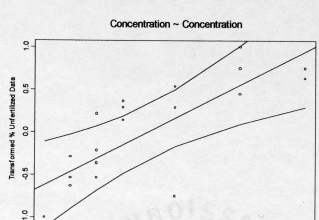
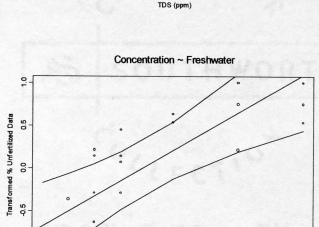


Figure B-3. Folded root transformed data from broodyear 2001 king salmon 24-hour fertilization trial (08/03/01-08/04/01, 7.9°). Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.





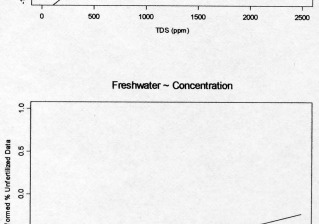


Figure B-4. Folded root transformed data from broodyear 2001 pink salmon 24-hour fertilization trial (08/13/01-08/14/01, 8.1°). Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

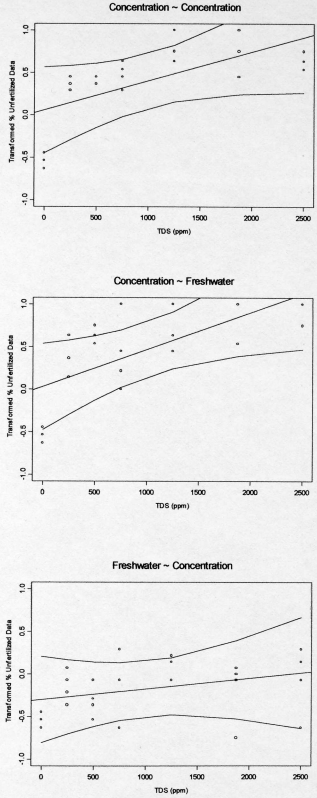


Figure B-5. Folded root transformed data from broodyear 2001 coho salmon 24-hour fertilization trial (10/24/01-10/25/01, 6.3°). Plots show a linear regression on and scatter plot of transformed data $\pm$ 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.

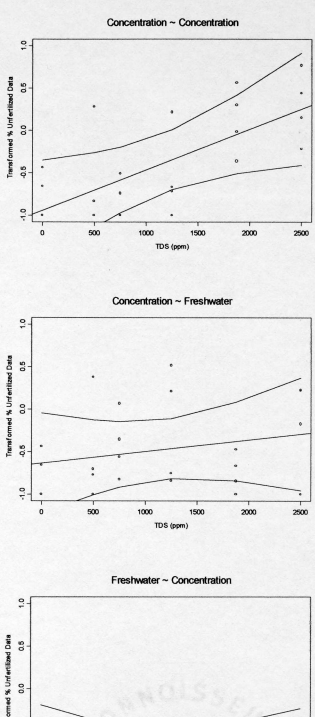


Figure B-6. Folded root transformed data from broodyear 2001 arctic char 24-hour fertilization trial (11/10/01-11/12/01, 4.5°). Plots show a linear regression on and scatter plot of transformed data ± 95% confidence bands. Concentration-concentration = eggs fertilized in test solution (~2 minutes) and transferred to the same concentration test solution. Concentration-freshwater = eggs fertilized in the test solution (~ 2 minutes) and transferred to control water. Freshwater-concentration = eggs fertilized in freshwater (~ 2 minutes) and transferred to test solution.