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Salmon as a Bioassay Model of Effects of Total Dissolved Solids

For: Alaska Science and Technology Foundation
4500 Diplomacy Drive, Suite 515
Anchorage, AK 99508

By: Michael S. Stekoll, William W. Smoker, Ivan A. Wang, and Barbi J. Failor
University of Alaska Fairbanks
Juneau Center School of Fisheries and Ocean Sciences
11120 Glacier Highway
Juneau, AK 99801

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Signature:_____
Principal Investigator Date

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I. Project Introduction

A. Executive Summary

We studied the lethal and sublethal effects of exposing salmonid juveniles to total dissolved solids (TDS). The need for this test came from two State of Alaska regulatory agencies (Department of Fish and Game and Department of Environmental Conservation) working in concert with industry's Alaska Council of Producers to revise discharge limits for total dissolved solids in the Alaska mining industry. Little work had been done previously on the effects of TDS on salmonids, particularly in Alaska, and their health, growth and development were regarded as an appropriate biological indicator of water quality.

We found that for short (24- to 96-hour) exposures, fertilization was the most sensitive stage to TDS exposure. We observed reduced fertilization rates in concentrations of TDS as low as 250 ppm. Natural background levels of TDS in our control water (Salmon Creek, Juneau) typically ranged from 20 to 60 ppm. We also observed differences in sensitivity to TDS at fertilization between species of salmonids. King, pink, and coho salmon were most sensitive, and Arctic char were least sensitive. Chronic, continuous, exposures to TDS revealed that fertilization and hatch were stages of development vulnerable to long-term TDS exposure.

B. Background

The goal of this research was to detect the effects of exposing Pacific salmon embryos to total dissolved solids (TDS) in fresh water. Embryonic salmon, found throughout freshwater ecosystems of Alaska, are physiologically sensitive to pollutants,

and have been shown to be adversely affected in later life by exposure as embryos to sublethal concentrations of pollutants (Heintz et al. 1999). Salmon are also readily cultured in the laboratory and are therefore an appropriate biological model for assaying effects of pollutants. Our plan was to expose coho salmon (*Oncorhynchus kisutch*) and other salmonid embryos to various concentrations of TDS and to observe immediate and latent effects in growth and development. This study design was modeled after the "whole life cycle" approach taken by NOAA Fisheries Auke Bay Laboratory for studying effects of hydrocarbon pollutants on fitness of Pacific salmon. We modified the "whole life cycle" approach by not releasing treated fish to complete their life cycle at sea. Instead, their growth and development were observed in controlled laboratory settings during the first (freshwater) part of their life cycles. The bioassays we developed will be useful for studying effects of other pollutants.

Our research with salmon was designed to complement work on laboratory bioassays by Dr. Lawrence Duffy (University of Alaska Fairbanks Department of Chemistry and Biochemistry). Dr. Duffy, Dr. Phyllis Weber-Scannell (Alaska Department of Fish and Game, Fairbanks) and their colleagues used Microtox (bacterial) and algal bioassays with TDS exposure. To facilitate comparisons, we used the same TDS composition as Drs. Duffy and Weber-Scannell. Our TDS composition is modeled after a specific field situation, Teck Cominco Alaska Incorporated's (TCAI's) Red Dog Mine near Kotzebue, Alaska. This TDS composition was developed by Maria Falutsu, formerly of TCAI (Mark Thompson, TCAI, and Phyllis Weber-Scannell, ADF&G, pers. comm.).

C. Approach

Our approach was twofold: 1) acute (96 hours or less) exposures of developing embryos to TDS at various stages of development and 2) chronic (continuous) TDS exposures of developing embryos.

D. Study Site

This research was conducted at the UAF Macaulay Salmon Laboratory in Juneau, Alaska. The Macaulay Salmon Lab is located in the Macaulay Salmon Hatchery, owned and operated by Douglas Island Pink and Chum, Inc. (DIPAC), a private non-profit salmon hatchery.

II. Acute Assays

A. Acute Exposures of Salmonid Embryos to Total Dissolved Solids

1. Abstract

Coho salmon (*O. kisutch*) were exposed to solutions of total dissolved solids (TDS) during several life stages of development from fertilization through button-up (emergence). TDS solutions from 250 to 2500 ppm (saturation point of this TDS composition) were modeled after Red Dog Mine effluent. Embryos and juveniles at various stages of development were exposed for 96 hours to determine acute response to TDS. Following the 96-hour exposures, the fish were cultured to completion of embryonic development to assess delayed effects from exposure. We observed four effects of the exposures at fertilization: short-term mortality, long-term mortality, the proportion of unfertilized eggs, and cumulative mortalities. Exposures at the other developmental stages were assessed for both immediate and delayed mortalities. Only reduced rates of fertilization with increasing concentration of TDS were significant.

2. Introduction

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 prevent TDS mixtures entering the aquatic ecosystem from causing adverse effects to aquatic life.

3. Methods

The experimental animals used were coho (*O. kisutch*) salmon. Broodstock were obtained as mature fish returning to Douglas Island Pink and Chum Inc.'s (DIPAC) Macaulay Salmon Hatchery located in Juneau, Alaska. Gametes from ten female and ten male coho salmon (*O. kisutch*) were used in the bioassays. Euthanized females were spawned by excision (Stekoll et al. 2002, Piper *et al.* 1986) into separate one-gallon polyethylene bags. The bags were then sealed and placed over ice until fertilized within three hours. Euthanized males were spawned (Piper *et al.* 1986) separately into waxed paper cups to monitor quality (excessive fluids in the milt caused us to discard the sample). The cups were then placed in separate one-quart polyethylene bags and placed over ice for less than three hours. In the laboratory, the eggs were pooled in a disinfected plastic bowl, and the milt was pooled in a disinfected 200 mL plastic cup.

In Broodyear 1999 (BY99, defined as the calendar year which the fish were fertilized), ninety-six hour bioassays were performed at the pre-determined developmental stages of fertilization, epiboly, eyed, hatch, and button-up (emergence, Piper *et al.* 1986). Since developmental rates of salmonids are temperature dependent, we measured temperatures daily. We used these temperatures and J.O.T. Jensen's Salmon Incubation Program version 1.3 for Windows, Department of Fisheries and Oceans, Nanaimo, BC, Canada, to predict the time to the desired development stage. The Broodyear 2000 (BY00) 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 test 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 Continuous Exposure (CE) 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 Fertilization Exposure (FE) treatment consisted of eggs fertilized in the test solution for two minutes and then transferred to control (Macaulay Salmon Hatchery) water. In the Post Fertilization Exposure (PFE) 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 one-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 0 ppm TDS added (Macaulay Salmon Hatchery) to a 2500 ppm TDS added 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 osmotic pressure of the highest TDS solution used.

In the 96-hour static water assay, eggs were placed in approximately one L of test solution in aerated one-L 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, about four weeks after fertilization. Water quality indicators, DO, pH, TDS, and temperature, were measured daily.

Solutions in the exposure chambers were changed after 48 hours. The solution was decanted to minimize disturbance to the eggs. The exposure solution was replaced by

pouring fresh test 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. Preserved eggs/embryos were dissected and examined under a 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 not counted in observations of developmental stages later than fertilization.

Table 2.1. Composition of 2,500 ppm TDS solution. The mixture models the outflow from the Red Dog Mine in Northwestern Alaska. The "2500 NaCl equiv." is the osmotic control solution with the same osmotic pressure as the 2,500 ppm TDS solution.

2500 ppm soln	g/L	Moles	MW
$CaSO_4$ 1/2 H_2O	2.30	0.0316	145.14
Na_2SO_4	0.14	0.0028	142.04
$MgSO_4$	0.20	0.0032	120.37
KCl	0.02	0.0008	74.55
Total TDS added	2.52	0.0386	
2500 NaCl equiv.	1.10	0.0372	59.44

Effects of TDS on mortality and fertilization were analyzed by analysis of variance, and linear regression. We used Dunnett's test to compare multiple treatments where appropriate. Proportions (e.g. rates of mortality or of fertilization) were transformed by

folded-roots ($Y' = \sqrt{(Y)} - \sqrt{(1 - Y)}$, Tukey 1977). Results were considered significant at $p <$ 0.05.

4. Results

Coho salmon eggs exposed in 1999 to TDS during fertilization experienced both increased mortality after fertilization and decreased fertilization with increasing TDS concentration (Table 2.2). This result was seen in both the Fertilization Exposures and the Continuous Exposure treatments (Figures 2.1 and 2.2). We observed four different effects of exposures at fertilization: short-term mortality (mortalities that occurred during the 96-hour assay as a proportion of fertilized eggs), long-term mortality (cumulative mortality through the fry stage as a proportion of fertilized eggs), fertility (proportion of unfertilized eggs at completion of cleavage), and overall percent affected (cumulative mortalities through the fry stage inclusive of unfertilized eggs). There was no significant effect of TDS exposure on either short-term mortality or long-term mortality resulting from the Fertilization Exposure (Table 2.2). In both the Continuous Exposure and Fertilization Exposure treatments, there was a linear response of TDS concentration 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 Continuous Exposure and Fertilization Exposure treatments). However, of those eggs in the Continuous Exposure that were successfully fertilized, only ~5% remained alive at the button-up stage. Those eggs in the Fertilization 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 TDS controls.

The 96-hour bioassays with BY00 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 Continuous Exposure experiment, and added a Post Fertilization Exposure experiment. In the Continuous 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 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 Post Fertilization 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 TDS controls.

Measured pH and TDS did not change over time. Temperature ranged at most $\pm$ 0.5° C. Measured pHs were between 7.37 and 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-hour acute bioassays.

Table 2.2. Coho salmon. 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. CE = eggs fertilized in the test solution (two min.) and transferred to the same concentration. FE = eggs fertilized in the test solution (two min.) and transferred to control water. PFE = eggs fertilized in control water (two 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					
	FE			CE			CE			PFE		
	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 % affected	0.0000	1250	1875	0.0000	1250	1875	0.6670	2500*	-	0.9750	2500*	-

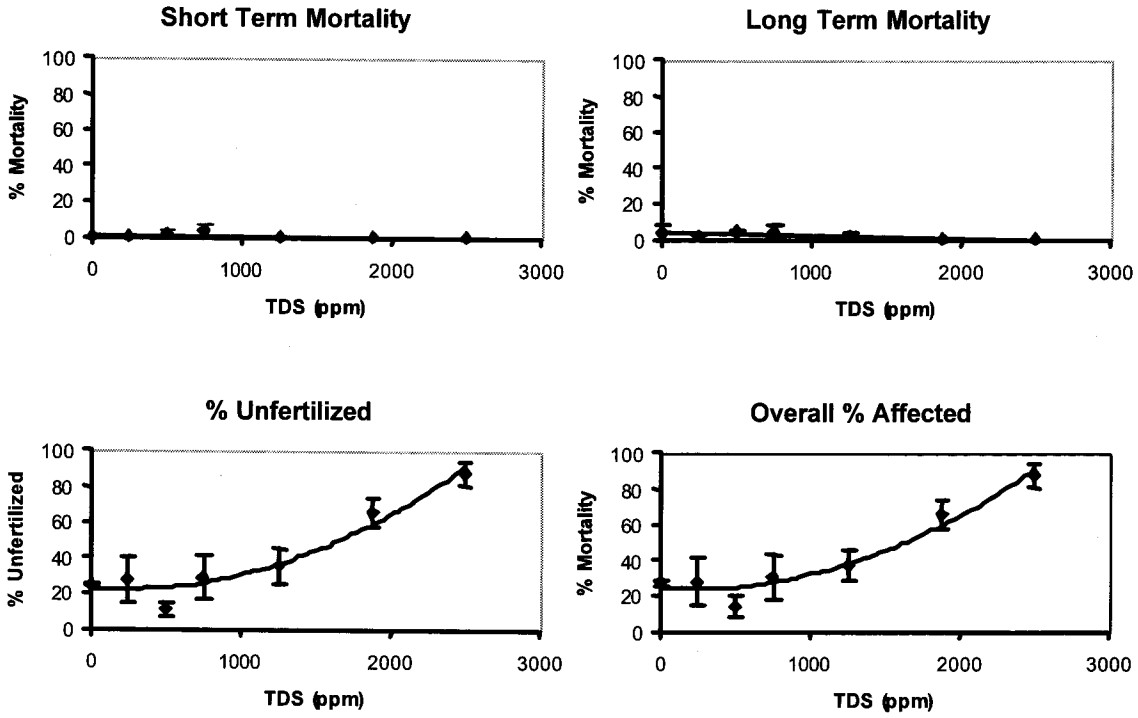


Figure 2.1. Coho salmon 96-hour acute bioassay at fertilization in BY99. Eggs were fertilized in concentrations of TDS (~two minutes) and moved to freshwater (FE). Untransformed data show mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Short-term mortality is 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 exclusive of unfertilized eggs. Overall percent affected is the cumulative mortality through button-up inclusive of unfertilized eggs.

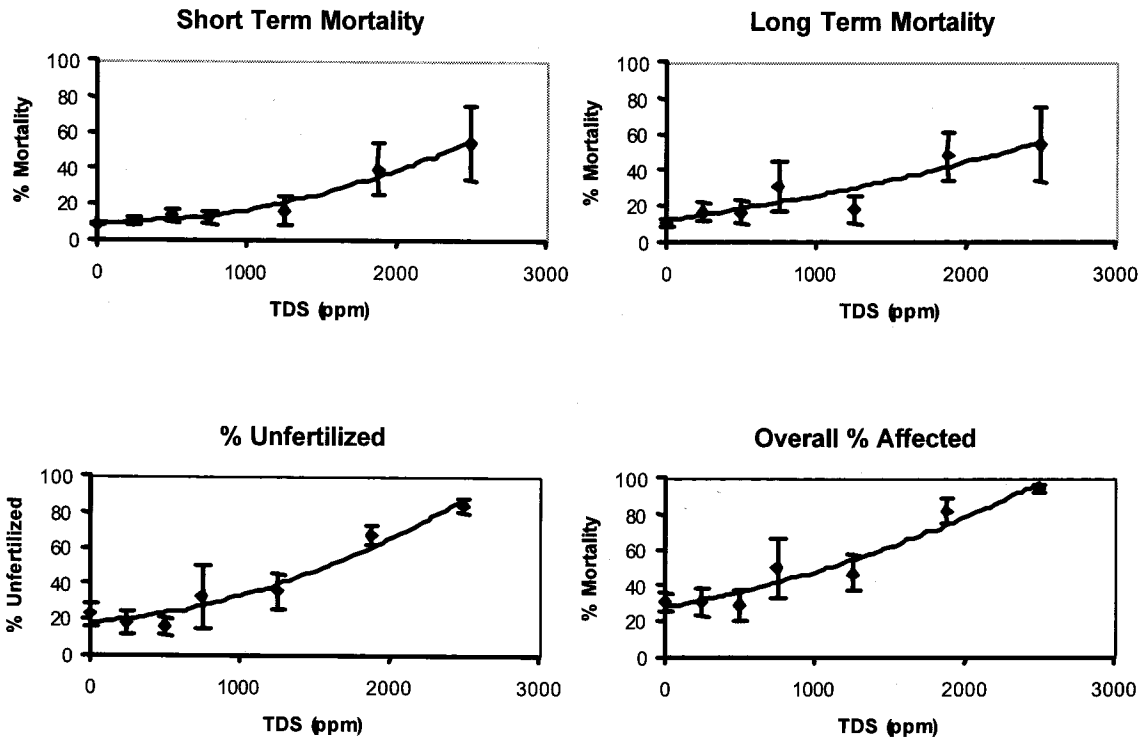


Figure 2.2. Coho salmon 96-hour acute bioassay at fertilization in BY99. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure (CE). Untransformed data show mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Short-term mortality is 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.

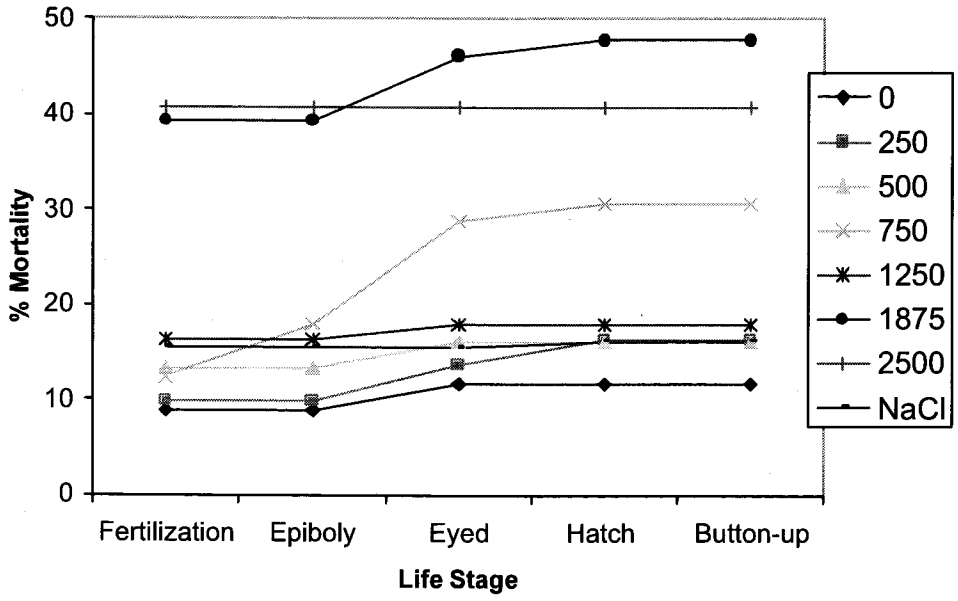


Figure 2.3. Coho salmon 96-hour acute bioassay at fertilization in BY99. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure (CE). Untransformed mortality data shows cumulative mortalities by concentration at specific stages of embryonic and juvenile development.

Table 2.3. Coho salmon. 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 Dunnett'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*

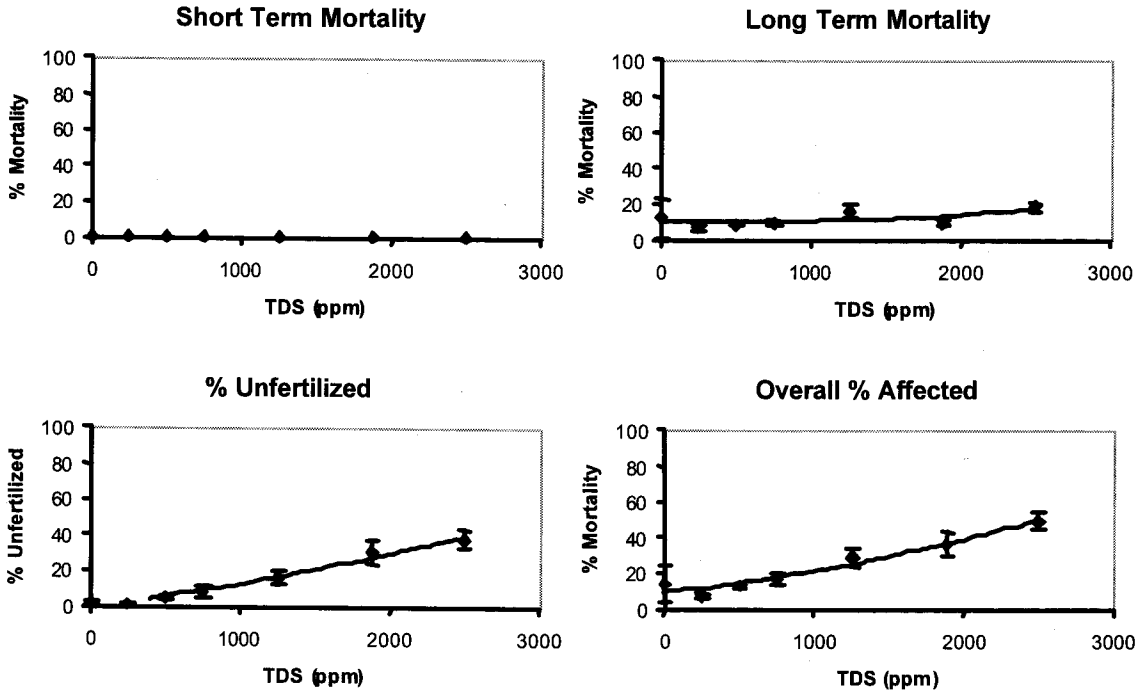


Figure 2.4. Coho salmon 96-hour acute bioassay at fertilization in BY00. Eggs were fertilized in and remained in concentrations of TDS for the 96-hour exposure (CE). Untransformed data show mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. Short-term mortality is 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.

5. Discussion

Salmonid exposure to high levels of TDS has yielded 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- to 3-hour exposure) in exposure solutions then grown out through eye-up in incubation stacks. Ketola *et al.'s* study most closely resembles our BY00 PFE 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.* examined the water chemistry of natural systems and ours explored a simulated industrial effluent. A comparison of results of the two studies shows them to be inconsistent.

Chapman *et al.* (2000) exposed both chironomid (*Chironomus tentans*) larvae and embryonic and juvenile rainbow trout (*O. 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 PFE 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 in TDS had more problems afterward. Eggs exposed at fertilization tended to survive to the eyed stage, but those exposed to the higher concentrations (1875 and 2500 ppm TDS) had high mortality rates between the eyed and alevin stages (Figure 2.3). In the 2500 ppm concentration in BY99 there was 50 percent mortality of the 50 percent that had been fertilized.

A separate 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 (Brannock *et al.* 2002). These ions were tested individually at levels equivalent to our 2500 ppm 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 2500 ppm 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

Brannock *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.

6. Conclusions

TDS toxicity is dependent upon both the composition and concentration of individual ions. Toxicity cannot be assessed simply based on a TDS measurement in mg/L. TDS occurs naturally in watershed systems, but anthropogenic sources of TDS are of 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 (Part II Section B). One possibility for research would be to compare two or several different populations of the same species, one wild and one cultured population, or several populations from different water sources and test what differences arise. When discharge limits are being considered, individual ions and compounds found in discharges should be tested with at least one salmonid species. Ideally, the salmonids would be from populations local to the discharge site being studied.

B. Comparative Short-Term Toxicity Tests Between Salmonids

1. Abstract

We developed a method for performing short-term (24-hour) embryo toxicity studies during salmonid egg fertilization. The assay we 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: 1) during only the first two minutes of fertilization 2) at two minutes after addition of sperm and 3) continuously from the addition of sperm to the end of the assay. The endpoint for the assay is the completion 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%. The results suggest that the assay can be generalized across the species and may be useful in setting site-specific criteria for discharging wastes.

2. Introduction

In this section, we describe 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 to TDS. The bioassays were run comparing the

response of six species of salmonids to a synthetic effluent that simulates outflows from the Red Dog Mine in northwestern Alaska.

3. Methods

The experimental animals of this project were coho (*Oncorhynchus kisutch*), chum (*O. keta*), king (*O. tschawytscha*), pink (*O. gorbuscha*), steelhead (*O. 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 at DIPAC's Macaulay Hatchery. Bioassays took place in the University wet lab in the Macaulay Hatchery. Temperature, pH, and TDS concentrations were measured prior to the beginning of each set of experiments. Since developmental rates of salmonids are temperature dependent, temperatures were measured before, during and at the end of the exposure time. We used these temperatures and J.O.T. Jensen's Salmon Incubation Program version 1.3 for Windows, Department of Fisheries and Oceans, Nanaimo, BC, Canada to predict the time to the desired development stage and decide when to end an experiment.

These bioassays were conducted in BY00 and BY01. For each bioassay three female and three male salmon of a single species were spawned. Gametes were

processed using the same procedure as in Part II Section A. 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: Continuous Exposure (fertilized in TDS solution and moved to same TDS, abbreviated CE), Fertilization Exposure (fertilized in TDS and moved to control water, abbreviated FE), and Post Fertilization Exposure (fertilized in control water and moved to TDS solution, abbreviated PFE). In addition the control consisted of fertilization in control water and then moved to control water. TDS test solutions ranged in concentration from the 0 ppm TDS added to Macaulay Salmon Hatchery water (control) to a 2500 ppm TDS added solution modeled after the outflow from the Red Dog mine near Kotzebue, Alaska (Table 2.1). There was also a sodium chloride osmotic treatment matching the osmotic pressure of the highest TDS solution used.

The Arctic char spawning differed a little from the above methodology in that the adult fishes were not sacrificed. Mature adults were anesthetized in MS-222 (methane-tricaine-sulfonate) and spawned by the live fish method described in Piper *et al.* (1986). From that point, the methodology continued as described for the other species.

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 one-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 (proxy for TDS levels) and temperature were recorded for all treatments.

All species tested were exposed from fertilization until they reached the 4- or 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 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 $p < 0.05$.

4. Results

In the Continuous Exposure experiment all species tested showed a significant trend of increasing numbers of unfertilized eggs with increasing TDS concentration

exposure (Figures 2.5-10, Table 2.5). 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 Fertilization Exposure experiment, chum, steelhead, king, pink, and coho salmon showed increased numbers of unfertilized eggs with increasing TDS concentration exposures (Figures 2.5-2.10, Table 2.5). The char were not affected even at the 2500-ppm level. This experiment however, did not show any groupings of species by response as observed in the CE 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).

In the PFE 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 2.6, Table 2.5). All other species tested showed no effect of TDS on the number of unfertilized eggs. Although coho salmon fertilization was significantly reduced at 750 ppm, fertilization success was not affected at the higher concentrations tested (Figure 2.9, Table 2.5).

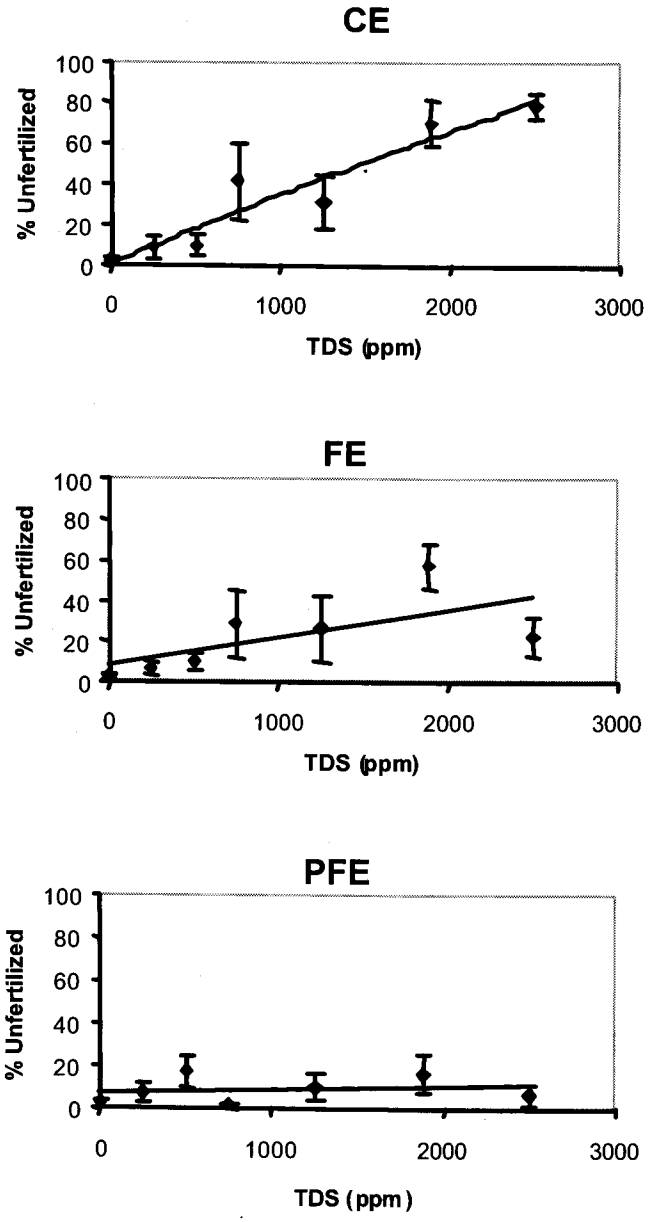


Figure 2.5. Chum salmon 24-hour fertilization trial (07/23/00-07/24/00, 8.5°) BY00. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.

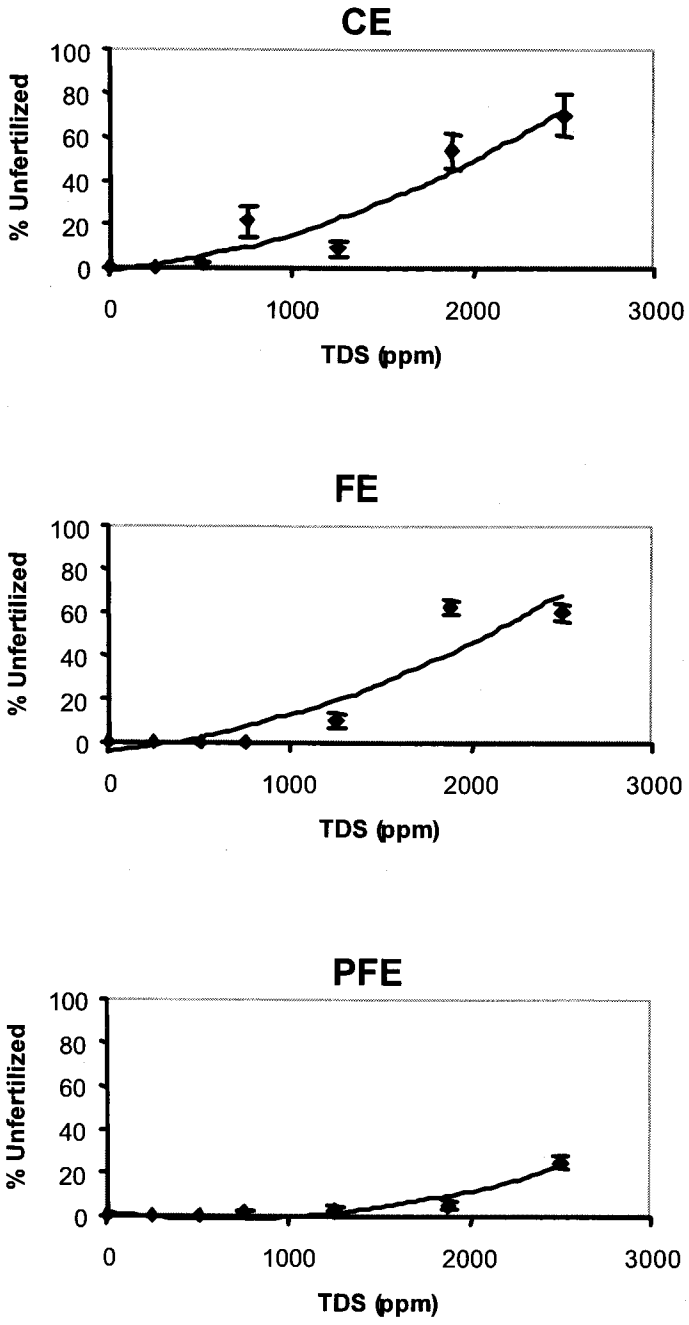


Figure 2.6. Steelhead 24-hour fertilization trial (05/25/01-05/26/01, 6.2°) BY01. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.

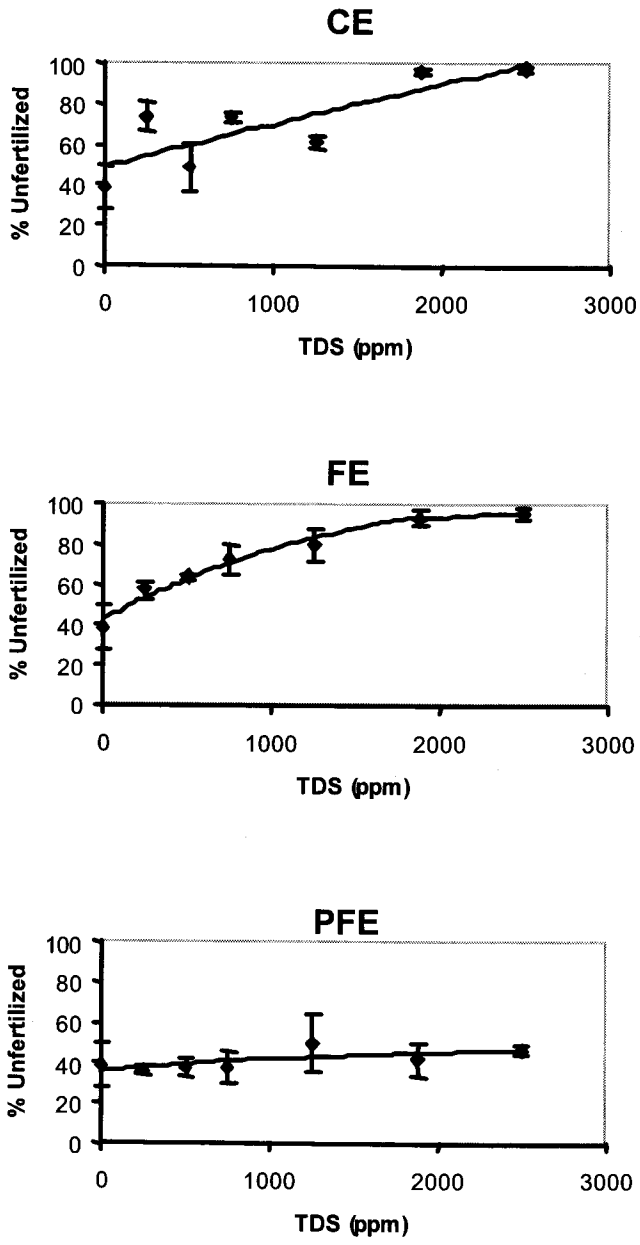


Figure 2.7. King salmon 24-hour fertilization trial (08/03/01-08/04/01, 7.9°) BY01. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.

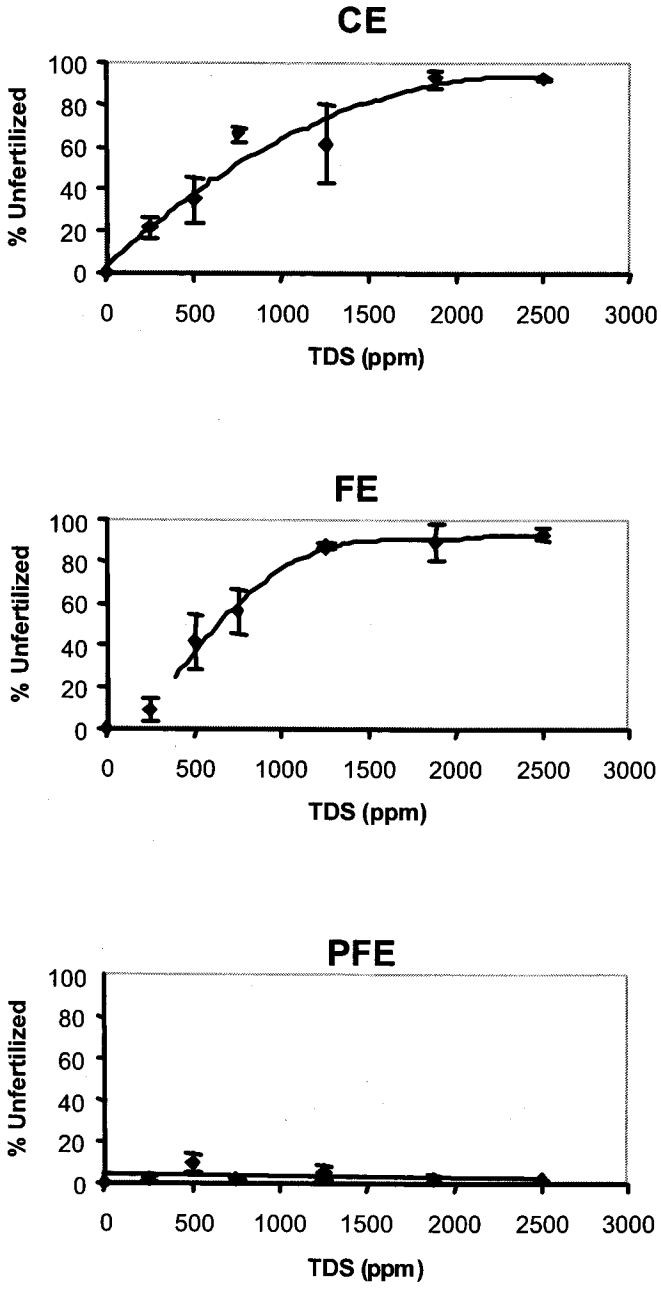


Figure 2.8. Pink salmon 24-hour fertilization trial (08/13/01-08/14/01, 8.1°) in BY01. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.

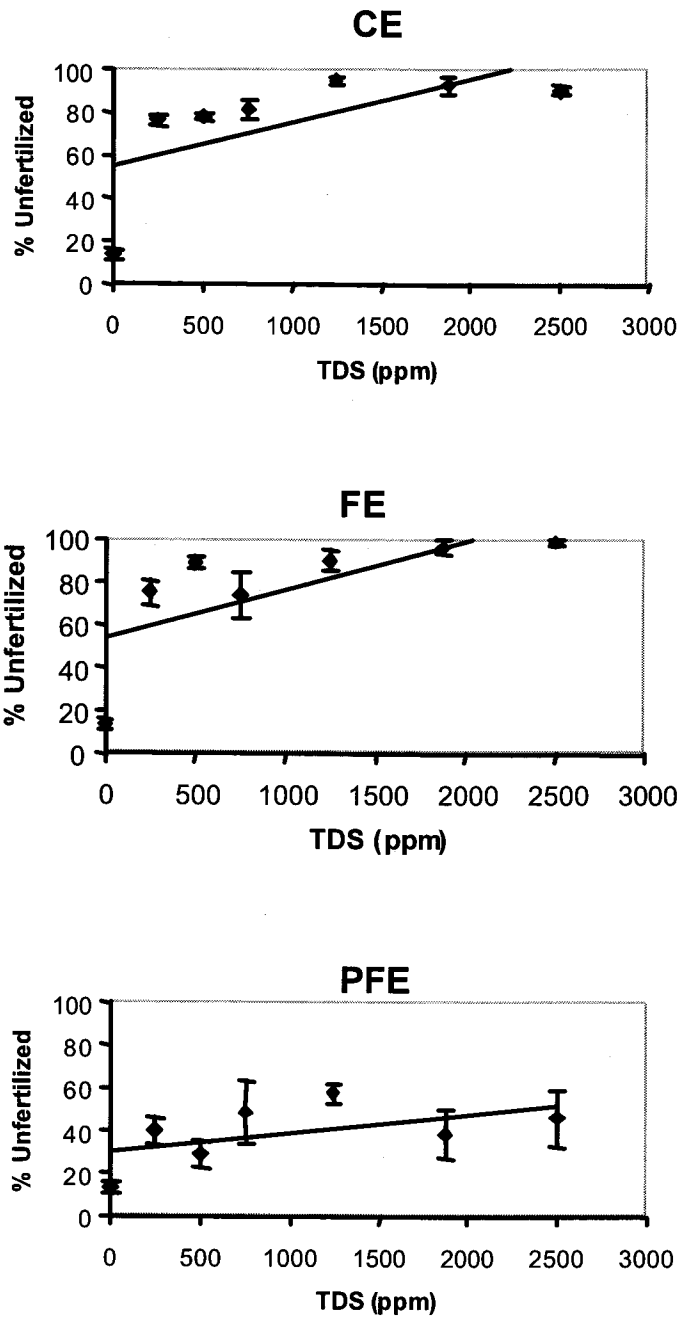
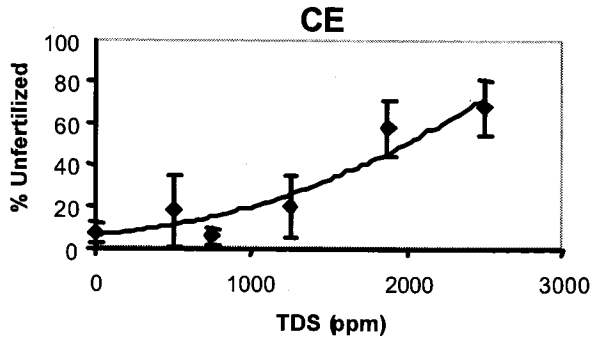
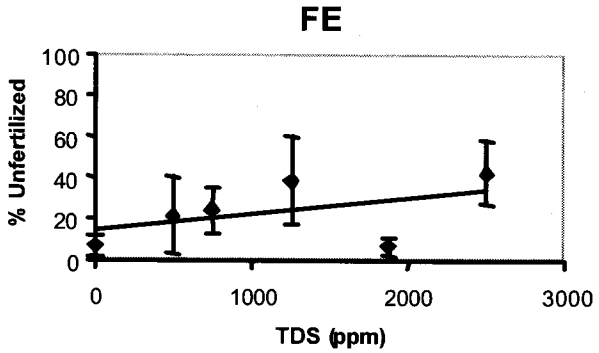


Figure 2.9. Coho salmon 24-hour fertilization trial (10/24/01-10/25/01, 6.3°) in BY01. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.





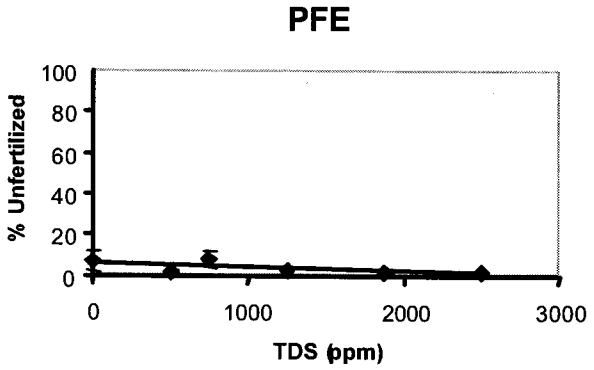


Figure 2.10. Arctic char 24-hour fertilization trial (11/10/01-11/12/01, 4.5°) in BY01. Untransformed data shows mean percentages of unfertilized eggs in samples ± standard error bars and a trend line. CE = eggs fertilized in test solution (~two minutes) and transferred to the same concentration test solution. FE = eggs fertilized in the test solution (~ two minutes) and transferred to control water. PFE = eggs fertilized in freshwater (~ two minutes) and transferred to test solution.

Table 2.5. Results of the three types of 24-hour fertilization trials for six species of fish. P-values are calculated from regressions on folded-root transformed data. CE = eggs fertilized in the test solution (two min.) and transferred to the same concentration. FE = eggs fertilized in the test solution (two min.) and transferred to control water. PFE= eggs fertilized in control water(two 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 one concentration higher not significantly different from control.

	CE			FE			PFE		
	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*	-

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 (data not shown).

While pH, and TDS measurements did not change between the beginning and end of an experiment, temperatures did change slightly. The water temperatures among the experiments varied (4.5-8.5°C) since these species of salmonids spawn at different times of the year, and we were using water near the ambient temperature (Table 2.6).

We were interested in exposing the eggs/embryos for the time from fertilization to the 4- or 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 developmental period. The time of exposure varied from 18 hours for pink salmon to 43 hours for Arctic char (Table 2.6).

Table 2.6. 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	9.0	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

5. 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. 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 (*O. 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 PFE study. With coho salmon, we found an LOEC of 750 mg/L, while Ketola *et al.* (1988) observed significant effects on survival to the eyed stage using calcium in excess of 520 mg/L. Part II Section A addresses the issue of grow-out of post exposure embryos. The BY00 PFE 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 Part II Section A found no differences in the 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. With 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 either to the same TDS solution or to control water. Thus, the experiment is one of reciprocal exposures: control-to-control, TDS to TDS (CE), control to TDS (PFE) and TDS to control (FE). 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 Part II Section A 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).

6. 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 toxicants and other species. The assay reported here is a relatively easy and inexpensive short-term bioassay that yields results quickly and

requires relatively little training. However, the assay does not 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 Part II Section A found significant effects on the long-term survival of embryos exposed at fertilization to this TDS mixture. Embryos that survived exposure at fertilization to the highest exposure concentration experienced 100% mortality by the button-up stage. Although the methodology described here will yield results quickly, there might be other effects of exposure to a toxicant that will not be seen.

III. Chronic Assays

A. Broodyear 1999

1. Introduction

The Broodyear 1999 (BY99) chronic TDS exposure assay was a pilot study. We wanted to determine the best way to test for the effects of chronic TDS exposure on juvenile salmon. Embryonic salmon require flowing water to remove metabolic waste during development. Flow through systems, as are commonly used in salmon incubation, would have been cost prohibitive to use in our study. In an attempt to contain costs of materials and labor, we opted for a batch-changing system.

2. Methods

In our batch-changing system for incubation, we made batches of the test solutions that were changed in all incubators at the same time. The frequency of batch changes was dictated by metabolic waste levels (ammonia). The maximum levels of ammonia and other water quality parameters were taken from the "Suggested water quality criteria for optimum health of salmonid fishes" in Fish Hatchery Management (Piper et al. 1986), except for solutes added to make the TDS solutions. Our incubators, one-liter polypropylene tri pour beakers with lids, were suspended in six 100-liter tanks (Figure 3.1).

Figure 3.1. BY99 incubators and water bath tanks

The tanks had hatchery water flowing through them to maintain incubator temperatures near ambient hatchery water temperature. Each tank held seven incubators, one for each treatment, for a total of 42 incubators. We tested five TDS concentrations: 1250, 750, 500, 250, and 125 ppm (equivalent to mg/L) added to hatchery water as well as the two controls: 1) straight hatchery water and 2) an osmotic control (sodium chloride solution with the same osmotic pressure as the highest TDS concentration)[1]. The composition of the highest concentration TDS solution as well as the NaCl solution is listed in Table 3.1. The highest concentration of TDS used in the chronic exposure (1250 mg/L) contained nearly twice the recommended upper limit for calcium in hatchery water

[1] Unless otherwise stated, the labels describing the solutions are ppm (mg/L) of chemicals added to the hatchery water. Background TDS readings and analysis of Macaulay Hatchery water were typically 20 to 60 ppm.

for salmon culture (Piper et al. 1986). Positions of the treatment solutions within a tank were randomly assigned.

Table 3.1. Solutes added to hatchery water to produce 1,250 ppm TDS solution and NaCl (osmotic equivalent of 1,250 ppm TDS solution) control solution for BY99.

Solute	g/L	Cation moles/L	Total moles/L
$CaSO_4 \cdot 1/2\ H_2O$	1.15	0.0079	0.0159
Na_2SO_4	0.07	0.0005	0.0009
$MgSO_4$	0.10	0.0008	0.0016
KCl	0.01	0.0002	0.0004
Total solutes added	1.33	0.0094	0.0186
NaCl control	0.55	0.0093	0.0186

On October 28, 1999, we collected eggs and milt from 10 female coho salmon and 10 male coho salmon from DIPAC's Macaulay Hatchery broodstock. The eggs and milt were taken at the outdoor raceways at Macaulay Hatchery, temporarily stored on ice, and transported to the laboratory. For each test beaker, approximately 200 eggs were fertilized in hatchery water, allowed to sit for two minutes, rinsed twice with 500 mL of hatchery water, and then poured into an incubator containing the test solution. Dead (white) eggs were picked out the day after fertilization and periodically afterwards.

Water quality measurements (pH, TDS, dissolved oxygen and temperature) were recorded for each incubator weekly during exposure. Ammonia levels were measured, using an Orion Model 95-12 ammonia electrode, for each incubator from two of the six tanks weekly. Water quality measurements were discontinued after the fish were moved to rearing tanks and placed in flowing water.

Starting on February 21, 2000, we estimated the rate of hatching for each incubator daily to the nearest 10% until hatching was finished. We also measured mortality rates, time to hatch, deformities, lengths and weights for possible effects of TDS exposure.

In April 2000, the number of alevins in each of the incubators was culled down to 25 individuals, to maintain ammonia at acceptable levels and to prevent crowding in the rearing containers. In late May/early June the fish reached the "button-up" fry stage where the abdominal cavity closed over the yolk sac and the fish required external food. After clipping, by design, exposure to TDS was discontinued by moving the fry from the one-liter static exposure incubators into 100-liter flow-through tanks. In order to identify to which treatment the fish were exposed, we clipped two fins from each fish for 14 different patterns, two for each treatment. Clip patterns were randomly assigned to the treatments. Lengths and weights were measured and recorded at the time the fish were clipped on May 31 and June 1, 2000.

Analyses for all of the Chronic Assays were done with SigmaStat version 2.03 for Windows, SPSS Inc., Chicago, IL. We ran one-way ANOVAs with Dunnett's multiple-pairwise comparisons with alpha = 0.05 unless otherwise stated. Standard errors and graphs were generated using Excel 2000 (SR-1) for Windows and Excel version 10 for Macintosh, Microsoft Inc., Redmond, WA.

We sent five sets of water samples for outside analysis to Columbia Analytical Services (CAS) in Anchorage, according to CAS's chain of custody procedures. We sent samples for basic analyses on December 29,1999, April 27, 2000, and August 7, 2000. On February 23, 2000 and June 6, 2000, we sent samples for more extensive analyses.

3. Results

a) Water Quality Measurements:

(1) TDS, DO, pH, Ammonia and Temperature

Except for ammonia levels being high on a few occasions, values of all variables remained within acceptable levels throughout the experiment. Ammonia measurements represent maximum values because they were taken just before water was changed. TDS measurements (extrapolated from conductivity) by probe were always lower than TDS analysis by evaporative technique. This result was due to calibrating our instruments with a factory-produced KCl standard, a solution less dense than the TDS composition we were using. DO levels were always near saturation. Measurements of pH ranged between 6.5 and 8.4 with moderate variation within an incubator from week to week.

Temperatures for the incubation and rearing containers in the chronic assay ranged between 2.1 and 8.8 °C. The incubators were within one °C of the hatchery water, but some warming occurred due to the air bubbling through the chambers.

(2) *Chemical Analysis*

Values for metals and other inorganics in all tested solutions were within the expected ranges (Tables 3.2a and 3.2b) except for magnesium and sodium in the June 2000 sample. We inadvertently substituted sodium sulfate for magnesium sulfate in the solution sent to CAS. We subsequently used a checklist for adding chemicals when making solutions. No ions exceeded the "Suggested water quality criteria for optimum health of salmonid fishes" or the "Suggested chemical values for hatchery water

supplies" (Piper et al. 1986) except for calcium. No organics (EPA Method 418.1) were detected in the February 2000 and June 2000 CAS samples.

b) Mortalities

We observed higher rates of cumulative mortalities from fertilization to hatching in the highest concentration, 1,250 ppm. The mortality rate averaged 29% for the 1,250 ppm, roughly twice all the other concentrations, although there was no statistically significant effect (Kruskal-Wallis ANOVA, P-value = 0.25). No significant differences, according to treatment, were seen for cumulative mortality rates from the eyed stage to the end of exposure, when fish would have emerged from gravel in the wild. There was high variation in cumulative mortality rates at emergence, between and within treatments, likely due to high ammonia levels observed on two days in some of the incubators. Rearing (post-button-up stage) mortalities were minimal and yielded no significant differences between groups.

c) Hatch timing

We saw a significant effect in hatch timing (P-value = 0.001, power = 0.93). Using Dunnett's Method of multiple comparisons versus controls, the highest concentration (1250 ppm) was significantly different from the 0 ppm controls. Time to midhatch averaged 15 days later for the highest concentration than the average of the controls.

Table 3.2a. CAS basic analysis of TDS exposure water for BY99. "-" denotes analyte not tested for in this sample. "ND" denotes not detected at method detection limits. "Unused" denotes water not previously used in the experiment. "Used" denotes water was a composite sample collected from incubators used to expose fish. "NaCl" is a sodium chloride solution with the same osmotic potential as the 1,250ppm TDS solution.

Date of sample	4/27/00	8/7/00	4/27/00	4/27/00	4/27/00	12/29/99	4/27/00	4/27/00	12/29/99	12/29/99	4/27/(
Concentration Sampled	0ppm used	0ppm unused	125ppm used	250ppm used	500ppm used	750ppm used	750ppm used	1,250ppm used	2,500ppm unused	2,500ppm used	NaC use
Total Metals (μ g/L)											
Aluminum	ND	ND	ND	ND	ND	-	ND	ND	-	-	
Calcium	8,600	6,760	41,500	74,000	139,000	188,000	201,000	325,000	600,000	572,000	8,(
Magnesium	989	664	3,050	5,120	9,180	11,900	13,300	21,500	40,500	38,500	9
Potassium	550	772	1,330	2,270	4,140	5,500	5,810	9,500	17,000	16,400	4
Sodium	650	427	2,850	4,960	9,560	13,600	13,900	23,100	42,200	41,000	220,0
Inorganic Parameters											
TDS (mg/L)	34	15	156	301	568	793	831	1,380	2,580	2,600	5
Alkalinity, Total as $CaCO_3$ (mg/L)	23	16	23	24	24	20	24	24	10	20	
Bicarb Alkalinity as $CaCO_3$ (mg/L)	23	16	23	24	24	20	24	24	10	20	
Nitrate as N (mg/L)	0.2	ND	0.3	0.2	0.2	0.1	0.2	0.2	0.1	0.1	
Chloride (mg/L)	0.7	0.4	1.2	1.6	3.1	5.2	4.6	7.0	13.3	13.8	3
Sulfate (mg/L)	7.7	5	98.5	183.5	341	455	525	1,201	1,610	1,610	

Table 3.2b. CAS analysis of TDS exposure water for BY99. "-" denotes not tested. "ND" denotes not detected at method detection limits. "Unused" denotes water not previously used in the experiment. "Used" denotes water from incubators. Antimony, Arsenic, Bismuth, Cadmium, Chromium, Cyanide, Fluoride, Nitrite, Petroleum Hydrocarbons (EPA Method 418.1), Lead, Mercury, Selenium, Silver, and Zinc were tested for but not detected. *In the 6/6/00 1,250 mg/L solution we accidently substituted sodium sulfate for magnesium sulfate.

Date of sample	2/23/00	6/6/00	2/23/00	6/6/00
Concentration	0ppm	0ppm	1250ppm	1250ppm
Sampled	unused	unused	unused	unused
Dissolved Metals (µg/L)				
Aluminum	-	ND	-	ND
Barium	45	45	49	47
Calcium	7,290	8,190	286,000	325,000
Copper	ND	ND	ND	10
Iron	ND	ND	ND	ND
Magnesium	792	934	17,400	*970
Manganese	ND	ND	6	14
Nickel	0.6	0.7	5.7	4.6
Potassium	846	950	8,050	10,000
Silicon	1,020	1,700	1,050	1,230
Sodium	492	490	21,300	*54,300
Total Metals (µg/L)				
Aluminum	-	ND	-	62
Barium	46	47	50	49
Calcium	7,350	8,140	289,000	333,000
Copper	ND	ND	ND	ND
Iron	105	111	131	152
Magnesium	798	965	17,900	*1,250
Manganese	10	23	20	33
Nickel	0.7	0.8	5.5	3.2
Potassium	864	940	8,160	10,100
Silicon	1040	1120	1160	1340
Sodium	501	530	21,300	*55,300
Cation-anion balance				
Cation sum (meq/L)	0.49	0.51	16.8	18.9
Anion sum (meq/L)	0.49	0.52	17.7	14.0
% diff cation-anion (meq/L)	0.2	1.4	2.5	14.8
Inorganic Parameters				
pH (pH units)	7.3	7.51	7.0	7.54
TDS (mg/L)	42	39	1,340	1,320
TSS (mg/L)	ND	ND	ND	15
Total settleable (mL/L/hr)	ND	ND	ND	ND
Turbidity (NTU)	0.6	0.9	3.7	2.3
Alkalinity, Total as CaCO3 (mg/L)	18	18	21	22
Bicarb Alkalinity as CaCO3 (mg/L)	18	18	21	22
Specific conductance (uMHOS/cm)	-	49	-	1,270
Ammonia as N (mg/L)	ND	ND	0.1	ND
Nitrate as N (mg/L)	0.1	0.2	0.1	0.3
Phosphorus (mg/L)	ND	ND	0.01	0.02
Chloride (mg/L)	0.4	0.5	6.2	7.5
Sulfate (mg/L)	5.5	6.5	820	642

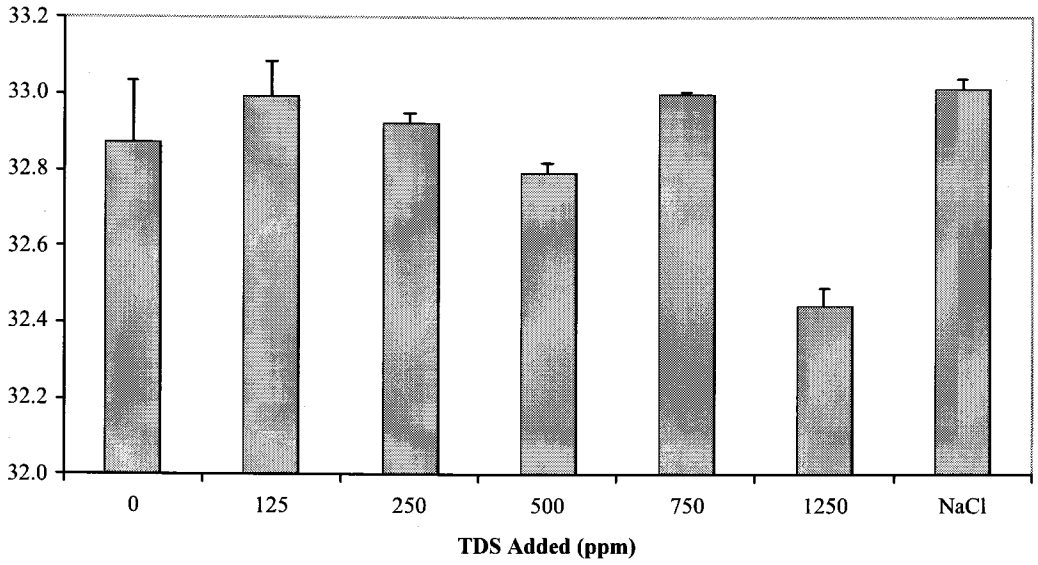
d) Deformities

While there were a few physical deformities observed, common to any experimental salmon incubation, there were no trends related to TDS exposure levels.

e) Lengths and Weights

When we clipped the fish in June 2000, we saw differences in lengths but not weights (Kruskal-Wallis ANOVA, P = 0.011 and 0.065 respectively). Although fish from the 1250-ppm group were shorter than other groups (Figure 3.2), the difference was not statistically significant. ANOVAs of final lengths and weights, taken in June 2001, were both statistically significant (P=0.010 and 0.014 and power = 0.687 and 0.636, respectively) but multiple comparisons did not show statistical significance between any concentration and the controls (0 ppm).

Figure 3.2. Length of BY99 Chronic TDS exposure fry at time of clipping, June 2000.



4. Discussion

The results of this broodyear were variable due, in part, to the experimental design. The differences we observed in mortality among treatments were not statistically significant (likely due to high variation between replicates). We experienced problems with high ammonia levels, possibly confounding our results. We believe the delayed hatching observed in the highest concentration of chronic TDS exposure for BY99 could have been caused by stress due to exposure to TDS, high ammonia levels, or an interaction between those factors. Delay in hatching at the highest TDS concentration was not seen in subsequent years' experiments.

Differences in hatch timing can reduce fitness in wild populations. In wild populations of coho salmon, high mortalities are observed during their early life history (Groot and Margolis 1991). The timing of hatching in salmonids is adapted to local weather conditions including freezing, flooding, and low water flow events. With hatch timing altered, higher than normal mortalities could be expected from salmon hatching at times other than ones to which they have adapted. Visible physical deformities were not an issue. With the uncertainty created by having temporary high ammonia levels as a cofactor in the analyses, conclusions from data from this broodyear should be made with caution.

B. Broodyear 2000

1. Introduction

We made two notable changes in the Broodyear 2000 (BY00) Chronic TDS exposure experiment. Due to difficulties we experienced with the static-water incubation system in the previous year, for BY00 we designed an incubation system using solutions dripping into the incubators to continuously flush out ammonia. Since in BY 1999 we did see differences at the highest concentration and little difference in the lower concentrations, we made added 2500 ppm TDS solution and dropped the 125 ppm TDS solution.

2. Methods

a) Incubation system

The incubation (Figure 3.3) chambers were made of 4" ABS plastic pipe with 3" PVC end caps glued flush with the bottom of the pipe. A nylon fitting was placed at the one-L mark to provide drainage below the top of the chamber. Lids were friction fit on top of the chambers with holes for the incoming airline, funnel for directing drips, and silicone rubber plug (access for water quality measurements). A guide was glued to the outside of the chamber to hold a pipet tip for delivery of the solutions via the funnel. Pipet tips were cleaned and replaced as needed. Inside the chamber was a disc of perforated plastic (1/8" diameter holes on 7/32" staggered centers) slightly smaller than the inside diameter of the chamber. The disc, where the eggs sat during incubation,

rested on the rim of the end cap. A 1/4" wide section of 4" PVC pipe with a 1/2" notch removed was sanded smooth and fitted over the disc to hold it in place. The center of the disc had a 1" hole to allow a 1" diameter rigid vinyl tube to fit through it. The rigid tube, housing the airline and air stone, was sized to touch the bottom of the chamber and come up to 1/4" below the water surface. Holes were drilled in the bottom of the rigid tubes to allow flow through to enable recirculation in the chambers. The chambers were suspended by a polystyrene support floating in the water bath (100 liter tank). Each replicate tank was fed by flowing hatchery water to maintain temperatures close to the hatchery water in the chambers. Each of the seven chambers in a replicate tank contained one of the five TDS concentrations: 2500, 1250, 750, 500, and 250 mg/L added to Macaulay Hatchery water or one of the two controls: 1) straight Macaulay Hatchery water and 2) a sodium chloride solution. The composition of the highest concentration TDS solution as well as the NaCl solution is listed in Table 3.3. The highest concentration of TDS used in the chronic exposure (2500 mg/L of added solutes) contained nearly four times the recommended upper limit for calcium in water for salmon culture (Piper et al. 1986). The test solutions were contained in five-gallon polyethylene carboys and distributed via nylon fittings, PVC pipes, and food-grade PVC tubing and were color coded by concentration. Plastic needle valves regulated drips, one for each of the 42 incubators. This Mariott-type delivery system was used to attempt to deliver a constant gravity feed rate. Initially, the drip rates were set to deliver one L per day. When the fish got near the hatching stage, we increased the flow rates to three L per day to provide more flushing of ammonia. Each day, the drip rates were checked and reset to deliver the proper rate.

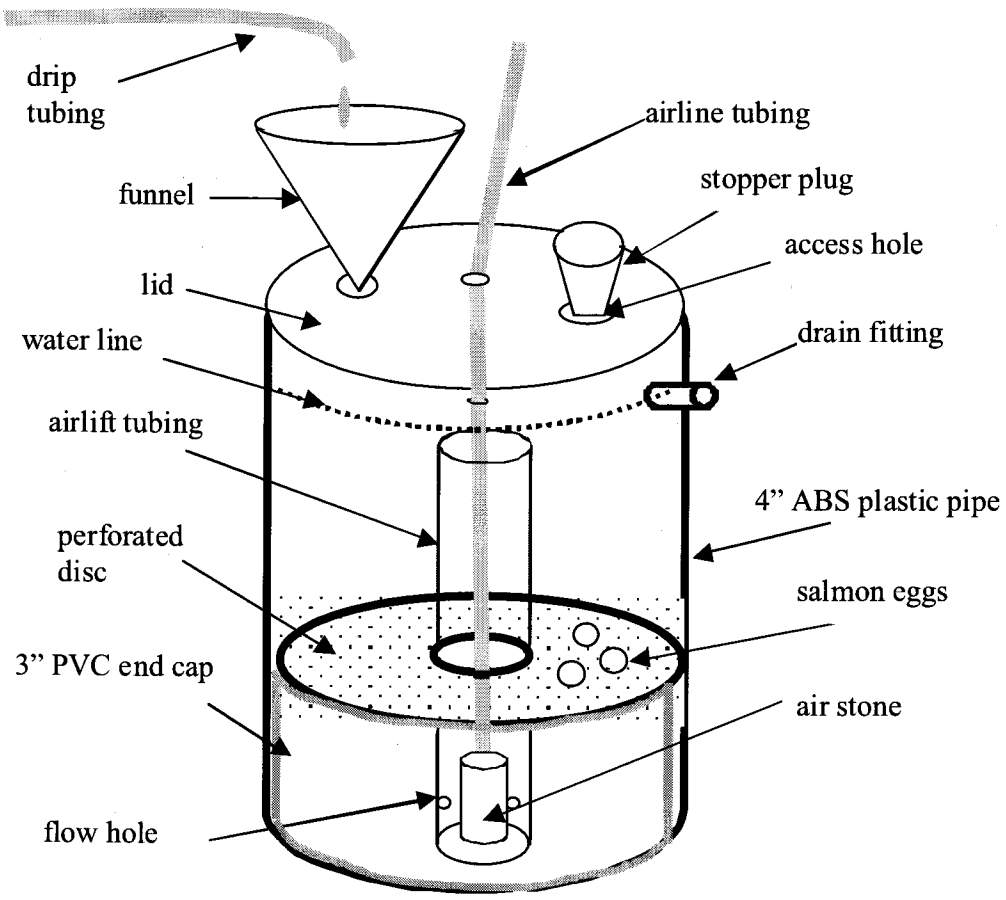


Figure 3.3. Schematic of incubation chamber

Table 3.3. Solutes added to hatchery water to produce 2,500 ppm TDS solution and NaCl (2500 ppm osmotic equivalent) control

Solute	g/L	Cation moles/L	Total moles/L
$CaSO_4 \cdot 1/2\ H_2O$	2.30	0.0159	0.0317
Na_2SO_4	0.13	0.0019	0.0028
$MgSO_4$	0.20	0.0016	0.0032
KCl	0.03	0.0004	0.0008
Total solutes added	2.66	0.0198	0.0386
NaCl control	1.10	0.0186	0.0372

b) Fertilization

Eggs were fertilized in the same solution to which they were exposed during the assay. Using a plastic spoon, approximately 50 eggs were placed in a four oz. (100 mL) wax-coated paper cup. Using a 1cc syringe, 0.2 mL of the pooled milt was added to each cup. Then 100 mL of solution were added to the containers to facilitate mixing of the milt and eggs. Eggs were allowed to sit for two minutes and then rinsed with test solution until clear (usually two rinses). The eggs were then poured into the appropriate chamber. This procedure was repeated for the other five replicate tanks.

c) Water Quality Measurements

(1) Conductivity, DO, pH, Ammonia and Temperature

We measured the same factors and frequency as in BY99. Temperatures in the reference incubator were recorded four times a day with an Onset Hobo® H8 Pro temperature data logger. Temperatures of every incubator were taken during the weekly water quality measurements.

(2) Chemical Analysis

Sets of water samples were sent on December 21, 2000, April 3, 2001 and May 14, 2001 to Columbia Analytical Services (CAS) in Kelso, WA, according to CAS's chain of custody procedures. The set submitted on December 21, 2000 contained two types of solutions, new Macaulay Hatchery water with no solutes added and a new 2,500 ppm TDS solution. The set submitted on April 3 contained seven concentrations of new and used solutions from the Chronic 2000 experiment. The April 3 set was analyzed for major ions only. The set submitted on May 14 contained samples of 1250 ppm used, 0 ppm new, and 1250 ppm new for full analysis.

In addition to analyses by CAS, we had two samples of Macaulay Hatchery (Salmon Creek) water analyzed for polyaromatic hydrocarbons (PAHs) using a low-density polyethylene (LDPE) method (NOAA 2001). The basis for this test is that LDPE strips used in a sampler absorb hydrocarbons from the water in which the sampler is deployed. The hydrocarbons absorbed by the strips can then be analyzed in a lab. The sampling device we used contained two LDPE strips to give an estimate of variability in sampling. For quality assurance, a method blank and two reference samples were used. The method blank and references were LDPE strips cleaned with methylene chloride and stored in the same way as the strips that were deployed. The reference strips were spiked with low levels of PAHs from a standard reference material. The samplers were deployed in a 600 L fiberglass tank (Frigid Units™ Model LS-700) with the flow set at approximately 7 L/min. The first sampler was deployed on March 7, 2001 and recovered on April 6, 2001 and a second was deployed on August 10, 2001 and recovered on September 7, 2001.

d) Hatch rates

Hatch rate observations were made daily starting one week before we estimated the fish would hatch. We recorded for each incubator to the nearest 10% how many eggs had hatched on a given day. The date when at least 50% of the eggs had hatched was defined as the midhatch date.

e) Mortality rates

Eggs that turned white before the eyed stage were carefully picked out, recorded, and preserved in Stockard's solution. At the eyed stage, all eggs were mechanically shocked by emptying each chamber of the solution and shaking each upright chamber horizontally approximately 10 cm three times. This procedure does not harm live embryos, but reveals dead eggs. The day after we shocked the eggs, dead eggs were picked, recorded, and preserved. Preserved dead eggs were examined with a magnifying lamp and/or a dissecting scope, to determine if an embryo had grown but died during exposure or if fertilization had never taken place. Dead alevin and fry were pulled out, preserved, and recorded daily until the experiment was ended, July 18, 2002.

f) Lengths and Weights

Lengths to the nearest millimeter and weights to the nearest hundredth of a gram were recorded for every fish at the time of fin clipping (April 20 and 21, 2001), for a random sample of twenty-five individuals per rearing tank on June 13, 2001, and for all surviving fish on April 15, 2002.

g) Histopathology

Two sets of fish were submitted to the Alaska Department of Fish and Game Pathology Lab in Juneau in June 2001. The first set was incubation mortalities (alevin) preserved during March and the second set was a sample of first-feeding fry that were alive just prior to the sample. The first set had 27 fish from the 2,500-ppm incubators and seven fish from the Macaulay Hatchery water incubators and the second set had four fish from Macaulay Hatchery water exposure and four from 1,250 ppm. Fish from the second set were anesthetized using MS-222. All fish were preserved in 10% buffered formalin.

3. Results

a) Incubation system

The gravity-fed system was a marked improvement in reducing ammonia concentratons compared to the static/batch system used in BY99. Mechanical shock of the embryos was also reduced with this system. The one weakness with this system was the fact that the drip rates did not stay consistent.

b) Water Quality Measurements

(1) Conductivity, DO, pH, Ammonia and Temperature

Conductivity measurements were in the expected range for a given concentration. Conductivity measurements within a given TDS concentration and within a given incubator were consistent from week to week. DO levels were at or near saturation (between 9 and 13 mg/L). The average pH for all incubators during the entire experiment

was 7.15 with the lowest observed value of 6.47 and the highest was 8.16. The average ammonia measurement for all incubators over the entire experiment was 0.00065 mg/L. The highest ammonia reading was 0.0081 mg/L, 65 percent of the recommended maximum of 0.0125 mg/L for juvenile salmonids (Piper et al. 1986). Temperatures for the incubation containers in the chronic assay ranged between 3.1 and 9.2 °C.

(2) Chemical Analysis

Values for metals and other inorganics were in expected ranges (Table 3.4a). No ions exceeded the "Suggested water quality criteria for optimum health of salmonid fishes" or the "Suggested chemical values for hatchery water supplies" (Piper et al. 1986) except for calcium. No organics were detected in the ranges tested in the 5/14/01 CAS samples.

The LDPE sample from March 7 to April 6 had "very low levels of PAHs and showed good precision and accuracy" according to Larry Holland, the chemist at NMFS Auke Bay Lab who performed the analysis. The sample from August 10 to September 7 showed slightly higher levels than in the previous period. The classes of PAHs with levels significantly higher than the method detection limits were C-2 napthalenes, C-3 napthalenes, and phenanthrenes (Table 3.4b)

Table 3.4a CAS analysis of TDS exposure water for BY99. "-" denotes analyte not tested for in this sample. "ND" denotes not detected at method detection limits. "Unused" denotes water not previously used in the experiment. "Used" denotes water was a composite sample collected from incubators used to expose fish. "NaCl" is a sodium chloride solution with the same osmotic potential as the 1,250ppm TDS solution.

Date of Sample Concentration Sampled	12/21/00 0ppm new	12/21/00 2500ppm new	4/3/01 0ppm used	4/3/01 250ppm used	4/3/01 500ppm used	4/3/01 750ppm used	4/3/01 1,250ppm used	4/3/01 2,500ppm used	4/3/01 NaCl used	5/14/01 0ppm new	5/14/01 1,250ppm new	5/14/01 1,250ppm used
Total Metals (µg/L)												
Aluminum	ND	53	-	-	-	-	-	-	-	79	73	50
Antimony	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Arsenic	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Barium	41	42	-	-	-	-	-	-	-	49	47	48
Bismuth	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Cadmium	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Calcium	6,930	614,000	8,680	72,500	143,000	204,000	362,000	478,000	8,210	9,760	326,000	316,000
Chromium	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Copper	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Iron	40	73	-	-	-	-	-	-	-	570	476	294
Lead	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Magnesium	772	36,900	928	4,520	8,670	12,200	21,600	35,000	872	1,040	20,100	19,100
Manganese	ND	25	-	-	-	-	-	-	-	138	137	136
Mercury	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Nickel	ND	3	-	-	-	-	-	-	-	ND	6	6
Potassium	827	18,400	693	2,390	4,320	6,060	11,100	20,600	714	1,060	9,580	9,000
Selenium	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Silicon	1,150	1,240	-	-	-	-	-	-	-	1,440	1,450	1,420
Silver	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Sodium	429	43,700	981	4,800	9,520	13,900	24,300	39,200	411,000	459	20,800	20,100
Zinc	ND	ND	-	-	-	-	-	-	-	17	ND	22
Cation-anion balance												
Cation sum (meq/L)	0.51	35.9	0.57	4.26	8.37	11.94	21.18	28.96	18.39	0.59	18.30	17.90
Anion sum (meq/L)	0.47	34.3	0.66	4.32	8.11	10.85	18.51	22.13	19.15	0.66	18.60	16.60
% diff cation-anion (meq/L)	4.1	2.2	7.1	0.8	1.6	4.8	6.7	13.4	2.0	5.90	0.84	3.96
Inorganic Parameters												
pH (pH units)	6.92	7.16	-	-	-	-	-	-	-	7.14	7.18	7.21
TDS (mg/L)	33	2,580	-	-	-	-	-	-	-	17	1,260	1,100
TSS (mg/L)	ND	7	-	-	-	-	-	-	-	ND	ND	<20
Total settleable (mL/L/hr)	ND	ND	-	-	-	-	-	-	-	ND	ND	-
Turbidity (NTU)	1.2	6.8	-	-	-	-	-	-	-	4.5	4.0	0.5
Alkalinity, Total as CaCO3 (mg/L)	16	21	-	-	-	-	-	-	-	20	21	24
Bicarb Alkalinity as CaCO3 (mg/L)	16	21	-	-	-	-	-	-	-	20	21	24
Specific conductance (uMHOS/cm)	49	2,590	-	-	-	-	-	-	-	71	1510	1530
Cyanide, Total (mg/L)	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Cyanide, WAD (mg/L)	ND	ND	-	-	-	-	-	-	-	ND	ND	ND
Fluoride (mg/L)	ND	<1.0	-	-	-	-	-	-	-	ND	<1	<1
Ammonia as N (mg/L)	ND	ND	-	-	-	-	-	-	-	ND	ND	0.82
Nitrate as N (mg/L)	0.2	0.6	0.3	0.4	0.4	0.7	0.7	1.3	0.7	0.3	0.7	0.7
Nitrite as N (mg/L)	ND	<0.5	-	-	-	-	-	-	-	ND	<0.5	<0.5
Phosphorus (mg/L)	ND	0.03	-	-	-	-	-	-	-	0.01	0.02	0.03
Chloride (mg/L)	0.4	13	1.2	1.8	3.0	4.4	7.1	13.2	657	0.6	6.5	6.3
Sulfate (mg/L)	5.0	1,610	8.1	185	365	494	858	1,020	7.2	11.1	864	762
Petroleum Hydrocarbons (µg/L)	-	-	-	-	-	-	-	-	-	ND	ND	ND

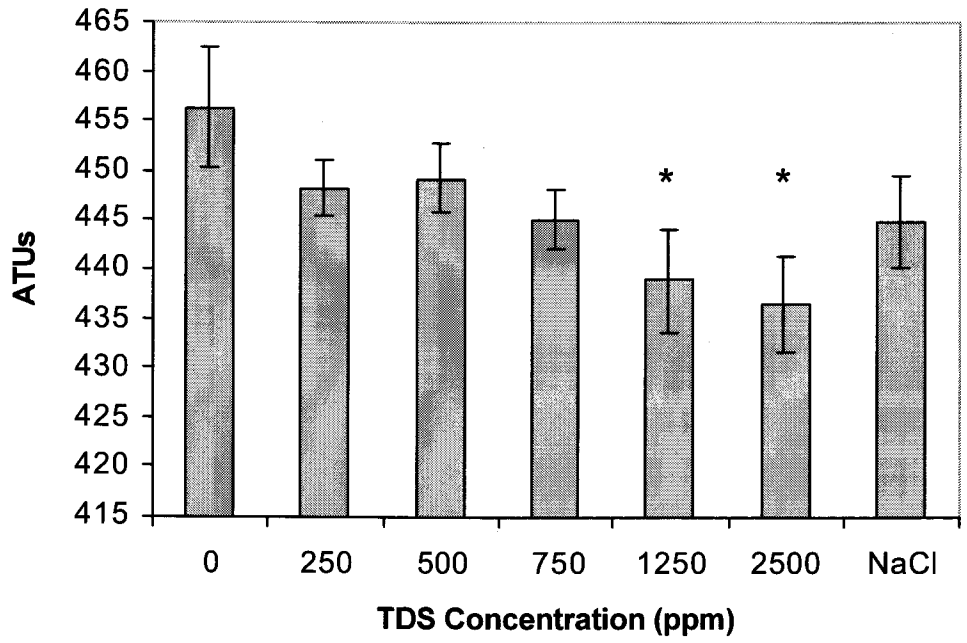
Table 3.4b. Polyaromatic Hydrocarbon analysis from NMFS Auke Bay Lab's LDPE sampling. Concentrations are ng/device. "ND" denotes not detected at Method Detection Limit. PAHs totals include analyte levels below the method detection limits.

Dates deployed	3/7/01 to 4/6/01		8/10/01 to 9/7/01	Method Detection Limit
Sample name	DIPAC-1	DIPAC-2	DIPAC-3&4 ave.	
naphthalene	ND	ND	ND	4.45
2-methylnaphthalene	ND	ND	ND	4.45
1-methylnaphthalene	ND	ND	ND	4.45
2,6-dimethylnaphthalene	**5.83**	**5.54**	**5.74**	2.63
C-2 naphthalenes	**26.39**	**22.87**	**24.48**	2.63
2,3,5-trimethylnaphthalene	**3.98**	**3.80**	**5.66**	1.59
C-3 naphthalenes	**37.37**	**31.73**	**69.45**	1.59
C-4 naphthalenes	**8.02**	**6.28**	**35.70**	1.59
biphenyl	ND	ND	ND	3.68
acenaphthylene	ND	ND	ND	0.92
acenaphthene	ND	ND	ND	3.03
fluorene	ND	ND	ND	3.79
C-1 fluorenes	**4.88**	**4.08**	**8.97**	3.79
C-2 fluorenes	ND	**3.85**	**8.04**	3.79
C-3 fluorenes	ND	ND	ND	3.79
dibenzothiophene	**1.72**	**1.55**	ND	1.22
C-1 dibenzothiophenes	**2.70**	**2.26**	**4.09**	1.22
C-2 dibenzothiophenes	**1.90**	**2.25**	**4.14**	1.22
C-3 dibenzothiophenes	**1.64**	**2.46**	**1.25**	1.22
phenanthrene	**18.96**	**17.61**	**17.44**	3.26
1-methylphenanthrene	**1.86**	**1.93**	**2.53**	1.09
C-1 phenanthrenes/anthracenes	**8.79**	**8.55**	**15.01**	1.09
C-2 phenanthrenes/anthracenes	**5.61**	**6.10**	**8.40**	1.09
C-3 phenanthrenes/anthracenes	ND	ND	**3.57**	1.09
C-4 phenanthrenes/anthracenes	ND	ND	ND	1.09
anthracene	ND	ND	ND	1.22
fluoranthene	**9.78**	**9.68**	**4.33**	2.68
pyrene	**7.80**	**6.32**	**7.28**	1.29
C-1 fluoranthenes/pyrenes	**2.97**	**3.44**	ND	1.29
benz-a-anthracene	ND	ND	**1.89**	0.44
chrysene	**3.24**	**3.26**	ND	1.59
C-1 chrysenes	ND	ND	ND	1.59
C-2 chrysenes	ND	ND	ND	1.59
C-3 chrysenes	ND	ND	ND	1.59
C-4 chrysenes	ND	ND	ND	1.59
benzo-b-fluoranthene	ND	ND	ND	3.29
benzo-k-fluoranthene	ND	ND	ND	3.00
benzo-e-pyrene	ND	ND	ND	4.89
benzo-a-pyrene	ND	ND	ND	3.03
perylene	ND	ND	ND	5.39
indeno-123-cd-pyrene	ND	ND	ND	8.74
dibenzo-a,h-anthracene	ND	ND	ND	7.16
benzo-g,h,i-perylene	ND	ND	ND	5.66
Total PAHs (ng)	**153.42**	**143.56**	**232.98**	

c) Hatch rates

Accumulated temperature units (ATUs, the sum of daily temperatures in °C) from fertilization to midhatch were significantly different (ANOVA) among treatments at a probability of 0.042. Dunnett's Test of multiple pairwise comparisons versus controls showed significant differences between the 2,500 and 1,250 ppm and the controls (0 ppm). That is, embryos in the two highest concentrations hatched earlier than the controls (Figure 3.4).

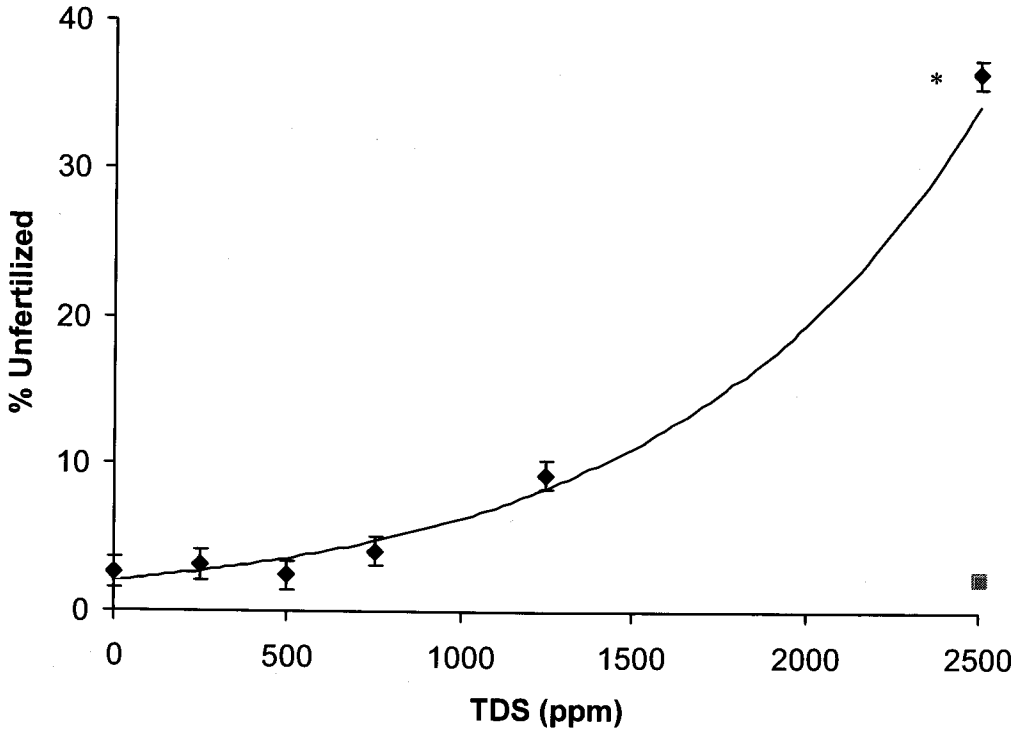
Figure 3.4. ATUs to hatch. Error bars are standard errors. Asterisks denote significant difference from 0 ppm, $p<0.05$.



d) Fertilization and Mortality rates

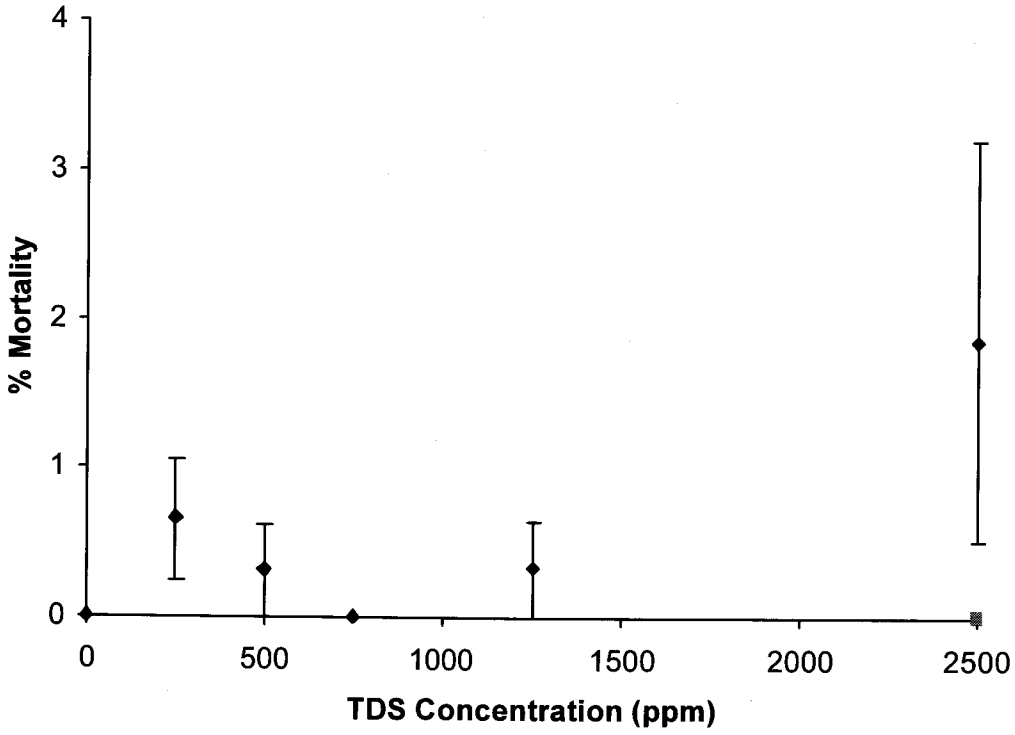
Eggs exposed to high levels of TDS during the fertilization process showed significantly lower fertilization success. The fertilization rates (Figure 3.5) were statistically significant at $p<0.001$ using empirical-logit transformation (Agresti) to improve the normality in the data. Dunnett's Test showed a significant difference between the 2,500 ppm and 0 ppm control.

Figure 3.5. Fertilization rates of Chronic BY00. Error bars are standard errors. The magenta square is the treatment with sodium chloride osmotic equivalent of 2,500 ppm. The curve is an exponential trendline. The astrisk denotes the treatment was significantly different from the 0 ppm controls.



Prehatch mortalities, eggs that were fertilized but died between fertilization and hatch (February 9) were low (under 2%) but statistically different (p=0.021) among the treatments using a ranked ANOVA (Figure 3.6). However, Dunnett's test did not detect a difference between treatments and the controls.

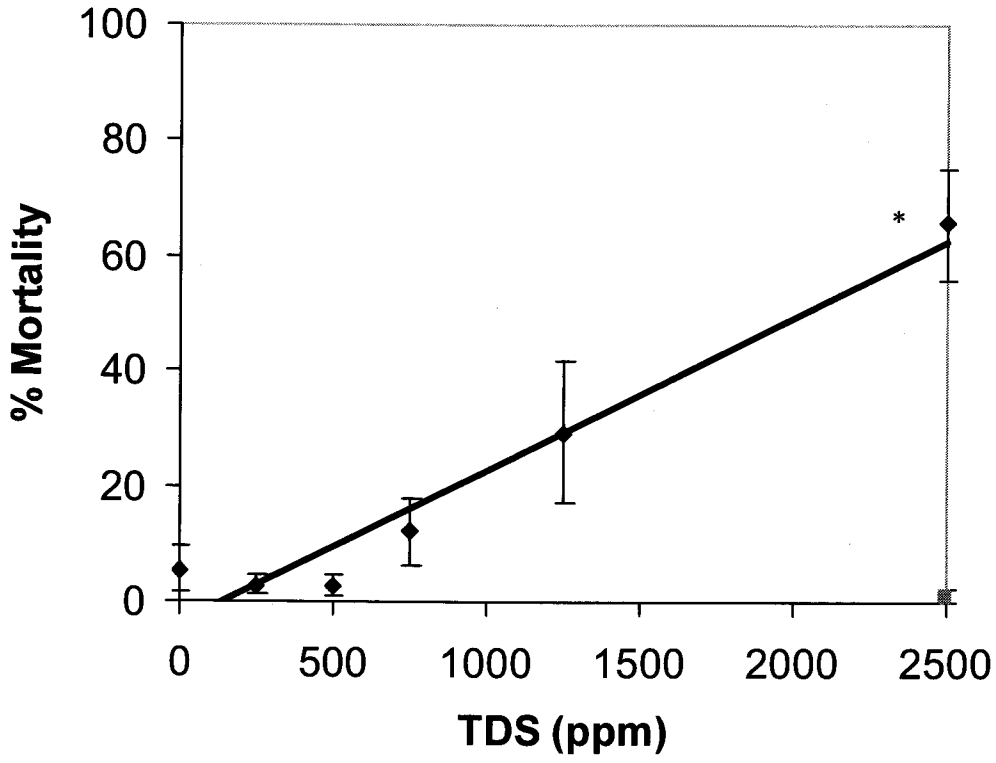
Figure 3.6. Chronic BY00 Prehatch mortality. Error bars are standard errors. The magenta square is the sodium chloride osmotic equivalent of 2,500 ppm.



Posthatch mortalities, from February 9 to March 31 (Figure 3.7), were significantly higher in TDS exposed fish (ANOVA : p<0.001, power = 1.00, linear

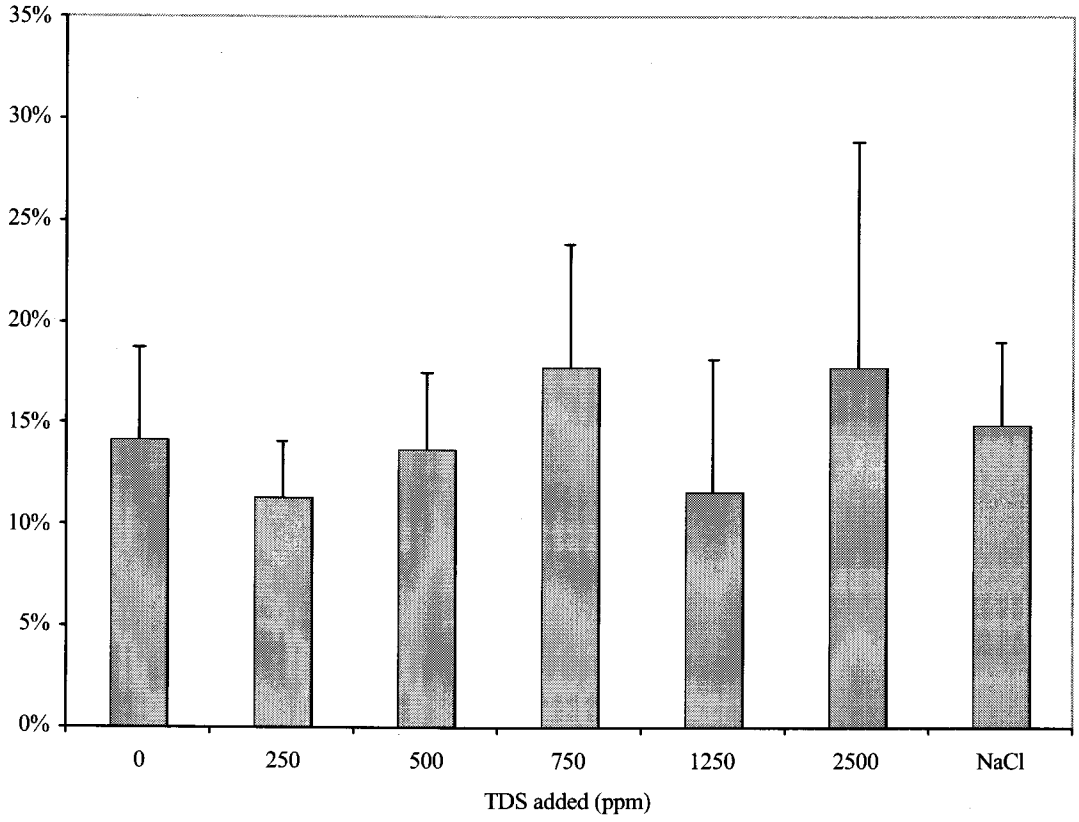
regression: slope = 0.0265, $p<0.001$, power = 1.00). Dunnett's Test showed a significant difference between the 2,500 ppm treatment and the 0 ppm control.

Figure 3.7. Chronic BY00 Posthatch mortality. Error bars are standard errors. The magenta square is the sodium chloride osmotic equivalent of 2,500 ppm. The asterisk denotes the treatment was significantly different from the 0 ppm controls.



Typical mortalities associated with first feeding were observed in the two months after rearing began. Minimal mortalities were seen over the next year. We did not see differences between treatments for cumulative rearing mortality rates over the entire period (Figure 3.8). Linear regression gave a p-value of 0.957, power = 0.028.

Figure 3.8. Chronic BY00 rearing mortality. April 28, 2001 to July 18, 2002.



e) Lengths and Weights

BY2000 Chronic exposed fish showed significant length and weight differences at clipping, April 20-21, 2001 (Table 3.5). The lengths ranged from 30.53mm for the 2500 ppm exposed fish to 34.18 for the 0 ppm controls, a difference of 12 percent. The weights ranged from 0.213g for the 2500 ppm exposed fish to 0.259g for the 0 ppm control, a difference of 22 percent. So at clipping the fish that had been exposed to high TDS were smaller.

Table 3.5. Chronic BY00 Lengths and Weights at time of clipping on April 20-21, 2001. P values for linear regressions (NaCl removed) and ANOVAs for lengths and weights were all <0.001 and power was 1.000 for both ANOVAs. Asterisks denote significantly different from 0 ppm controls.

Exposure Concentration	Average Total Length (mm)	Length Standard Error	Average Weight (g)	Weight Standard Error	Sample size
0 ppm	**34.18**	**0.09**	**0.259**	**0.002**	**174**
250 ppm	**34.17**	**0.10**	**0.259**	**0.002**	**172**
500 ppm	**33.57***	**0.11**	**0.251***	**0.002**	**171**
750 ppm	**33.43***	**0.11**	**0.243***	**0.003**	**159**
1250 ppm	**32.23***	**0.18**	**0.231***	**0.003**	**120**
2500 ppm	**30.53***	**0.37**	**0.213***	**0.007**	**30**
NaCl	**34.35**	**0.09**	**0.263**	**0.002**	**158**

For a sample taken on June 13, 2001, two months later we did not see statistically significant length and weight differences (Table 3.6). The lengths did show a superficial difference at this time. Lengths ranged from 37.50mm for the 2500 ppm exposed fish to 42.59mm for one of the 0 ppm control groups, a difference of 14 percent and the weights ranged from 0.42g for the 2500 ppm exposed fish to 0.64g for one of the 0 ppm control groups, a difference of 54 percent. Because we sampled 25 individuals from each tank, and there were far fewer fish exposed to 2500 ppm that survived to fry, we, by chance, sampled only two fish from the 2500 ppm group. It is likely that the non significant result in length differences is due to the low power (0.131 for length and 0.119 for weight) of the test, and not to a lack of effect.

Table 3.6. Chronic BY00 Lengths and Weights on June 13, 2001. P values of ANOVAs for lengths and weights were 0.263 and 0.281 and powers were 0.131 and 0.119 respectively.

Exposure Concentration	Average Total Length (mm)	Length Standard Error	Average Weight (g)	Weight Standard Error	Sample size
0 ppm- clip A	**41.50**	**1.47**	**0.59**	**0.08**	**12**
0 ppm- clip B	**42.59**	**0.92**	**0.64**	**0.06**	**17**
250 ppm	**41.55**	**1.08**	**0.59**	**0.06**	**22**
500 ppm	**43.76**	**0.96**	**0.70**	**0.06**	**17**
750 ppm	**41.20**	**0.82**	**0.56**	**0.04**	**15**
1250 ppm	**41.21**	**1.07**	**0.53**	**0.04**	**14**
2500 ppm	**37.50**	**4.50**	**0.42**	**0.20**	**2**
NaCl	**43.42**	**0.90**	**0.67**	**0.05**	**26**

On July 17 and 18, 2002 the lengths ranged from 121.0mm for the 2500 ppm exposed fish to 127.4mm for one of the 0 ppm control groups, a difference of 5 percent (Table 3.7). The weights ranged from 16.86g for the 2500 ppm exposed fish to 18.99g for one of the 0 ppm control groups, a difference of 13 percent. While the trend of high TDS-exposed fish being smaller was apparent, there were no statistically significant length and weight differences. The power of the tests, 0.123 for length and 0.050 for weight, were well below the desirable 0.800 level

Table 3.7. Chronic BY00 Lengths and Weights on July 17 and 18, 2002. P-values of ANOVAs for lengths and weights were 0.274 and 0.506 and powers were 0.123 and 0.050 respectively.

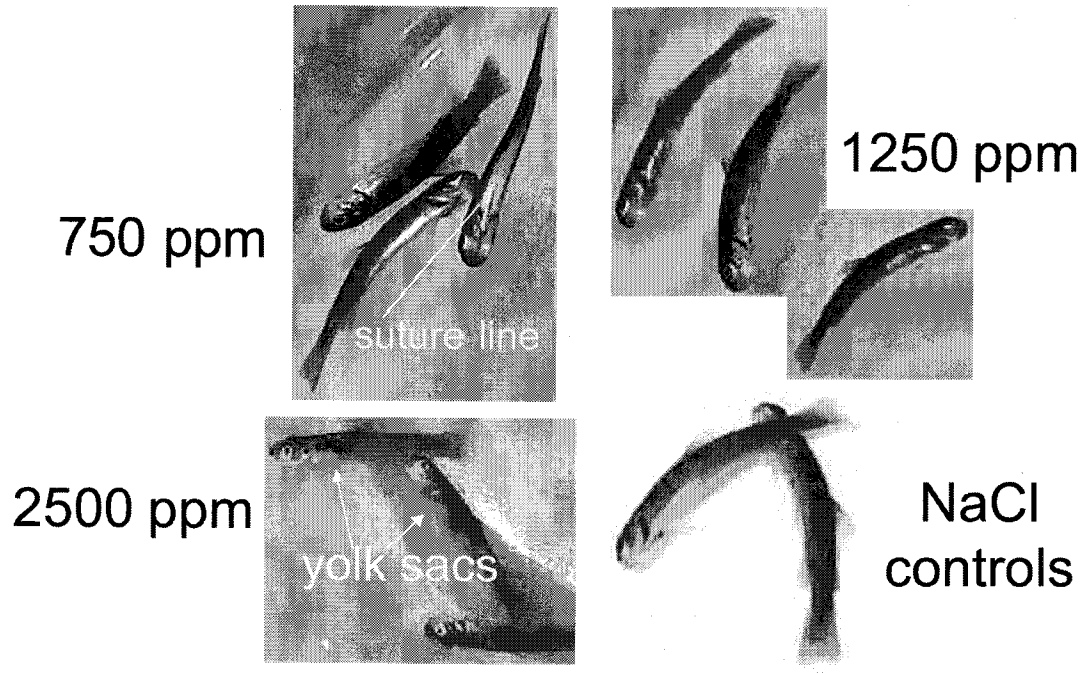
Exposure Concentration	Average Total Length (mm)	Length Standard Error	Average Weight (g)	Weight Standard Error	Sample size
0 ppm- clip A	**127.4**	**1.3**	**18.99**	**0.58**	**52**
0 ppm- clip B	**126.0**	**1.8**	**18.56**	**0.77**	**46**
250 ppm	**126.5**	**1.3**	**18.47**	**0.60**	**59**
500 ppm	**124.5**	**1.0**	**17.98**	**0.44**	**97**
750 ppm	**126.7**	**1.2**	**18.67**	**0.55**	**65**
1250 ppm	**123.4**	**1.2**	**17.29**	**0.46**	**55**
2500 ppm	**121.0**	**3.6**	**16.86**	**1.41**	**9**
NaCl	**125.5**	**0.9**	**18.27**	**0.44**	**106**

f) Histopathology

We saw two trends in the alevin mortalities. Many of the alevins were described as having mild to extreme spinal curvature and/or domed craniums. We saw no apparent relationship of deformities to TDS exposure concentration. We saw one qualitative trend related to TDS exposure concentration. Alevins exposed to higher TDS concentrations had more yolk than those incubated in hatchery water at the time of fin clipping (Figure 9). That is, the exposed fish appeared to be slower in development post hatch.

Only one trend was seen in the fry submitted. Severe exophthalmia (popeye) was noted for all fish.

Figure 3.9. BY00 fry at time of clipping, April 20, 2001. NaCl controls looked similar to 0 ppm controls, fully sutured and thin bellies. Abdominal suture lines are visible in 750 ppm. Bellies of 1250 ppm were covered with skin but bulging more than lower concentrations. 2500 ppm had yolk sacs visible.



4. Discussion

a) Water Quality Measurements

In spite of the difficulty in maintaining consistent solution flow rates, water quality measurements and analyses gave no indication that water quality, other than the experimental added dissolved solids, affected the results of the assay. The large amount of time involved in daily resetting valve flow rates and inconsistent flow rates observed, convinced us to use peristaltic pumps to deliver solutions for the following year.

The LDPE method results appear quantitative, but in truth are qualitative in nature. A rough estimate of the volume of water that flowed past the sampler during the

30-day deployment is 3,000 liters. Differences in laminar flow can affect results on a finer scale. Because we do not know the partition or equilibrium coefficients or the true flow rates past the devices, we cannot calculate the actual concentrations of the hydrocarbons that were in the water. At best, we can compare the values obtained with those from other systems. Compared to some other locations sampled using this method, these results are relatively low (Table 3.8). According to Larry Holland (pers. comm.), Lake Creek is considered a pristine stream with only airborne and minor residential sources of hydrocarbons. Lake Creek feeds into Auke Lake. Auke Lake is a roadside lake with hydrocarbon sources primarily from watercraft use in summer months.

Table 3.8 LDPE-PAH levels compared

Location	Season	# of days deployed	Total PAHs (ng/device)	Total PAHs per day
DIPAC #1	Spring	28	153	5.46
DIPAC #2	Spring	28	144	5.14
DIPAC #3&4	Summer	28	233	8.32
Lake Creek	Winter	21	12	0.57
Lake Creek	Summer	21	33	1.57
Auke Lake	Winter	21	300	14.29
Auke Lake	Summer	21	4,000	190.48

b) Hatch Rates

We observed earlier hatch times in the higher TDS concentrations. Environmental conditions like freezing, low oxygen, and toxins can cause salmon to hatch earlier than they would in conditions that are more favorable. In this experiment, higher levels of TDS were the environmental condition that caused hatching to occur earlier. This result is the opposite effect seen in the BY99 chronic TDS experiment and is likely due to the effect of ammonia in BY99, which was not an issue in BY00.

Differences in hatch timing can reduce fitness in wild populations. In wild populations of coho salmon, high mortalities are observed during their early life history (Groot and Margolis 1991). Salmon adapt to local weather conditions including freezing, flooding, and low water flow events. With hatch timing altered, higher than normal mortalities could be expected from salmon hatching at times other than ones they have adapted to over generations.

c) Fertilization and Mortality Rates

Fertilization is clearly the most sensitive stage for TDS exposure in juvenile coho salmon but not the only sensitive stage. We observed higher mortality rates within a week after hatching in fish exposed to the higher concentrations of TDS solutions. These findings imply that coho salmon exposed to high concentrations of TDS during incubation were harmed in a manner that is expressed shortly after hatching when fish are adjusting to being outside of their protective eggshells. They are challenged specifically by osmoregulation and metabolism changes.

d) Lengths and Weights

TDS caused a significant decrease in length and weight of exposed fish. Such a decrease at the time of swim up may have serious consequences in subsequent survival of these fish in their natural streams. Smaller fish may be less competitive in catching prey and more susceptible to being prey themselves.

There are three possible explanations for length and weight differences being highly significant at time of clipping but not significant less than two months later. The first explanation is, many of the smallest fish died between the times of the two samples. When the smaller fish were gone, length and weight differences were no longer

significant. The second explanation is that TDS exposure during incubation caused stress to the developing fish. When the TDS exposure was removed at the time of clipping, the fish incubated in higher concentrations were able to catch up in growth to their counterparts incubated in lower concentrations. The third explanation is we did not have sufficient power due to the low sample size from the highest concentration treatment. While the differences in length and weight were not statistically significant two months after TDS exposure was stopped, the differences did not disappear, especially in the highest concentration.

e) Histopathology

There are a number of causes of exophthalmia seen in the salmon fry, including physical trauma, infectious agents, toxins, and congenital defects. Since it was observed in every fish studied, whatever the cause, it was not related to TDS exposure concentration.

The spinal curvature and domed craniums observed in the alevin mortalities could be symptoms related to their causes of death or a natural post-mortem effect but these abnormalities were not linked to TDS-exposure concentration.

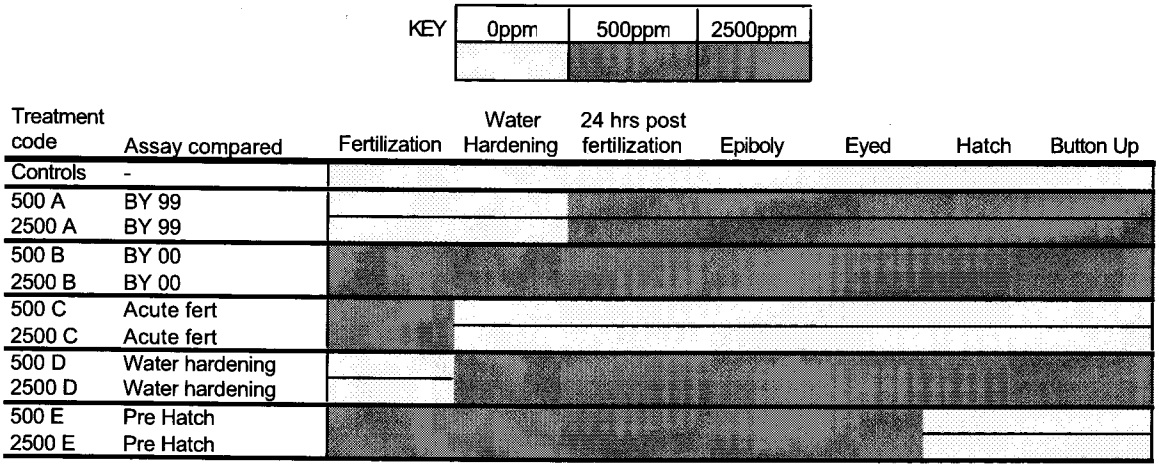
While we did not obtain quantitative data on yolk absorption, it is qualitatively clear that fish exposed to higher concentrations of TDS were slower in absorbing their yolks, in spite of hatching earlier than those fish exposed to lower concentrations. One component of an individual's fitness is outmigration timing. The time at which salmon in a particular stream emerge from the gravel and begin feeding is selected for over generations. Alteration of outmigration timing can cause a reduction in fitness.

C. Broodyear 2001

1. Introduction

There were two main differences in the Broodyear 2001 (BY01) experiments from the previous two years' experiments. First, we used peristaltic pumps instead of a gravity fed drip system in order to deliver solutions to the incubators more consistently and to reduce technician time involved in adjusting flow rates. Second, we altered the experimental design. For the experimental design, we wanted to tie together design features of the acute assays and the previous years' chronic TDS experiments. We also wanted to test whether the high posthatch mortalities we observed in past chronic experiments could be repeated if fish were exposed to TDS only before hatch, or whether fish must be exposed to TDS before, during, and after hatch. In order to accomplish this goal, we picked five exposure treatments to test (Figure 3.10): A) 24 hours after fertilization to button-up (similar to the BY99 experiment), B) fertilization to button-up (similar to the BY00 experiment), C) fertilization to two minutes post fertilization (similar to the acute fertilization assays), D) two minutes after fertilization to button-up (similar to the acute fertilization assays), and E) fertilization to hatch (testing cause of posthatch mortalities).

Figure 3.10. BY01 Treatment Scheme



2. Methods

a) Incubation system

We used the same incubators and water bath tanks in BY01 as were used in BY00. For this experiment, we used two TDS concentrations, 500 ppm and 2,500 ppm, based on the Red Dog Mine composition used in previous experiments. Each of the seven tanks had one control (Macaulay Hatchery water) incubator randomly assigned to it. Each of the five treatments had four replicates of each TDS concentrations, using forty incubators randomly assigned to the remaining openings, bringing the total number of incubators to forty-seven. For the solution delivery system, we used two Ismatec 40 Channel Pumps (Cole Parmer, Vernon Hills, IL), silicone (peroxide cured) three-stop tubing and extension tubing. Three 45-gallon polyethylene (FDA-approved) tanks (US Plastic Corp, Lima, OH) were used to hold the solutions. Solutions of 500 ppm and 2,500 ppm were made by adding calcium sulfate (Table 3.3) to Macaulay Hatchery water

and mixing with mixers fitted with Teflon-coated stirring rods overnight, then adding the remaining chemicals and stirring for two hours. We set the flow rates at 1L/day at the beginning of the experiment. We daily flushed the tubing by turning the pumps to maximum flow for one minute. Flow rates to each incubator were measured weekly. As designed, the fertilization to hatch (treatment E) had exposure solutions switched to control water on February 22, 2002, before hatching began.

b) Fertilization

We used the same methods as in BY00 except the solution in which the eggs were fertilized and the solution in the incubator in which they were subsequently placed may or may not have been the same, depending the assigned treatment.

c) Water Quality Measurements

(1) Conductivity, DO, pH, Ammonia and Temperature

The same parameters and intervals were used as in BY00. To keep ammonia levels low, we doubled the solution drip rates on February 22, 2002, before hatching began.

(2) Chemical Analysis

Two sets of water samples were sent to Columbia Analytical Services (CAS) in Kelso, WA, according to CAS's chain of custody procedures. The first was taken and sent on October 26, 2001 and the second on March 27, 2002. The set submitted on October 26 was analyzed for major ions in three types of solutions: unused Macaulay

Hatchery water with no solutes added, an unused TDS solution with 2500 ppm of solutes added and a TDS solution with 2500 ppm of solutes added used in the Chronic 2001 experiment (composite sample from all incubators with 2500 ppm solution) and for BTEX and Diesel range of hydrocarbons for unused Macaulay Hatchery water with no solutes added. The set submitted on March 27 was analyzed for major ions only for three types of solutions: unused Macaulay Hatchery water with no solutes added, an unused TDS solution with 500 ppm of solutes added and an unused TDS solution with 2500 ppm of solutes added. Using the same methods as in the prior year, NMFS Auke Bay Laboratory ran a PAH analysis on one sampler set on February 13, 2002 and recovered March 13, 2002.

d) Hatch rates

We used the same methods as in BY00 (Part III, Section B).

e) Mortality rates

We used the same methods as in BY00. Three incubators were removed from the analyses: the reference incubator (a control incubator with the temperature probe and continuous TDS monitor) and two other control incubators that had large air bubbles under the perforated disc (which caused high mortalities).

f) Lengths and Weights/Rearing

We used the same methods as in BY00 (Part III, Section B). We recorded lengths and weights for every fish at the time of fin clipping (May 24, 2002) and at the end of the experiment on July 30, 2002. We clipped the fish with either the upper or lower caudal lobe and either the right or left ventral fin. We reared them in five tanks

with each rearing tank holding one of the 0 ppm control incubators (the two control incubators with air bubbles under the eggs were eliminated from the experiment), one of the 500 ppm treatments, and one of the 2,500 ppm treatment, eighty fish from each.

g) Histopathology

One set of fish was submitted to the Alaska Department of Fish and Game Pathology Lab in Juneau in April 2002. This set consisted of alevin that were alive just prior to taking the sample. The sample had six fish incubated in 2500 ppm the entire experiment (three fish each from two of the 2,500 ppm incubators) and six fish incubated in plain hatchery water (three fish each from two of the 0 ppm incubators). The fish were preserved in 10% buffered formalin. The Pathology Lab used 6μ sections with hematoxylin and eosin stains.

3. Results

a) Incubation system

We observed some clogging of the intake ends of the tubing by a mix of crystals/precipitate, diatoms, and organic matter in December 2001. We subsequently cleared the tips a few times a week. Throughout the experiment, in spite of the clogging, the flow rates to each incubator were consistently at the target rates ± 10%.

b) Water Quality Measurements

(1) Conductivity, DO, pH, Ammonia and Temperature

Conductivity measurements were in the expected range for a given concentration. Conductivity measurements within a given TDS concentration and within a given incubator were consistent from week to week. DO levels were at or near saturation (between 10 and 13 mg/L). The average pH for all incubators during the entire experiment was 6.95 with the lowest observed value of 5.98 and the highest was 8.02. The average ammonia measurement for all incubators over the entire experiment was 0.00025 mg/L. The highest ammonia reading in any incubator during the whole experiment was 0.0025 mg/L, 19 percent of the recommended maximum of 0.0125mg/L for juvenile salmonids (Piper et al. 1986). Temperatures for the incubation containers in the chronic assay ranged between 1.7 and 6.5 °C with no differences between exposure treatments.

(2) Chemical Analysis

Values for metals and other inorganics were in expected ranges (Table 3.9a). No ions exceeded the "Suggested water quality criteria for optimum health of salmonid fishes" or the "Suggested chemical values for hatchery water supplies" (Piper et al. 1986) except for calcium. No organics were detected in the ranges tested in the October 26, 2001 CAS samples.

Lower total PAHs were found in the February to March 2002 sample than in samples taken in the previous years (Table 3.9b).

Table 3.9a. CAS analysis for BY01 samples. "-" denotes not tested for and "ND" denotes not detected at method detection limits.

Date of sample	10/26/01	10/26/01	10/26/01	3/27/02	3/27/02	3/27/02
Concentration Sampled	0ppm new	2,500ppm new	2,500ppm used	0ppm new	500ppm new	2,500ppm new
Dissolved Metals (mg/L)						
Calcium	6,770	587,000	588,000	8,510	139,000	632,000
Magnesium	701	36,400	36,500	955	8,880	41,100
Potassium	ND	17,000	17,600	ND	3,840	19,000
Sodium	439	40,600	41,700	501	9,640	45,900
Total Metals (mg/L)						
Calcium	6,960	606,000	585,000	8,820	140,000	649,000
Magnesium	713	38,600	36,100	993	8,960	42,700
Potassium	ND	17,700	17,100	ND	3,800	19,100
Sodium	538	42,200	40,700	498	9,710	45,900
Inorganic Parameters (mg/L)						
Chloride	0.5	12.1	15.3			
Sulfate	5.4	1630	1690			
Nitrate as N	0.3	2.3	2.3			
BTEX						
Benzene	ND					
Toluene	ND					
Ethylbenzene	ND					
m,p-Xylenes	ND					
o-Xylene	ND					
Diesel Range Organics						
C10 - C25	ND					

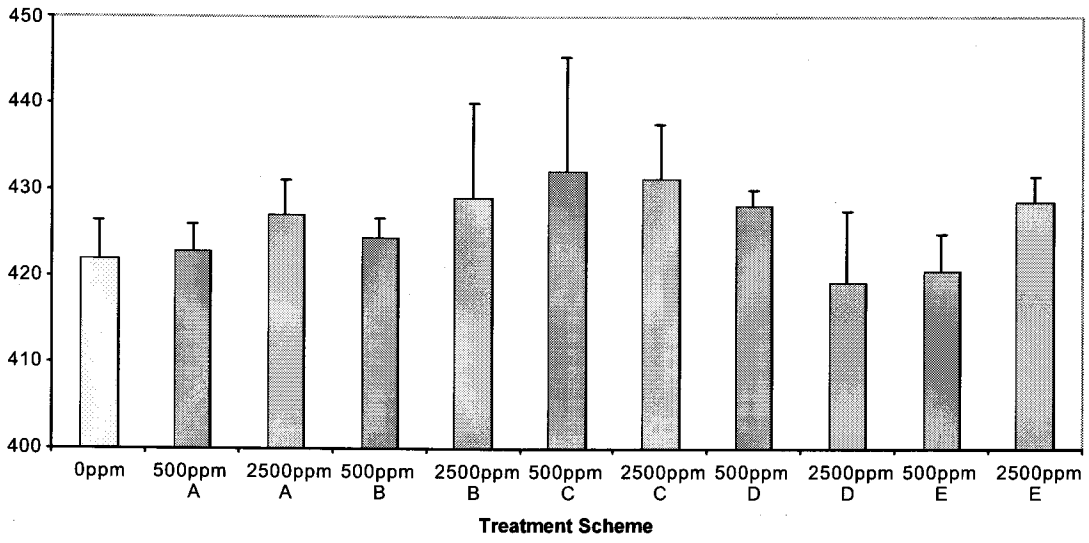
Table 3.9b. Polyaromatic Hydrocarbon analysis from NMFS Auke Bay Lab's LDPE sampling. Concentrations are ng/device. "ND" denotes not detected at Method Detection Limit. PAHs totals include analyte levels below the method detection limits.

Dates deployed	2/13/02 to 3/13/02		Method Detection
Sample name	Strip-1	Strip-2	Limit
naphthalene	5.32	9.43	4.45
2-methylnaphthalene	ND	8.27	4.45
1-methylnaphthalene	ND	ND	4.45
2,6-dimethylnaphthalene	3.38	3.63	2.63
C-2 naphthalenes	11.82	13.56	2.63
2,3,5-trimethylnaphthalene	2.16	3.42	1.59
C-3 naphthalenes	14.67	18.59	1.59
C-4 naphthalenes	5.25	5.00	1.59
biphenyl	ND	ND	3.68
acenaphthylene	ND	ND	0.92
acenaphthene	ND	ND	3.03
fluorene	ND	7.66	3.79
C-1 fluorenes	7.27	10.53	3.79
C-2 fluorenes	ND	4.11	3.79
C-3 fluorenes	ND	ND	3.79
dibenzothiophene	ND	ND	1.22
C-1 dibenzothiophenes	1.59	ND	1.22
C-2 dibenzothiophenes	ND	ND	1.22
C-3 dibenzothiophenes	ND	ND	1.22
phenanthrene	10.54	9.78	3.26
1-methylphenanthrene	ND	ND	1.09
C-1 phenanthrenes/anthracenes	6.90	5.50	1.09
C-2 phenanthrenes/anthracenes	3.86	3.78	1.09
C-3 phenanthrenes/anthracenes	2.01	1.31	1.09
C-4 phenanthrenes/anthracenes	ND	ND	1.09
anthracene	ND	ND	1.22
fluoranthene	5.61	5.38	2.68
pyrene	2.56	1.49	1.29
C-1 fluoranthenes/pyrenes	1.75	1.66	1.29
benz-a-anthracene	ND	ND	0.44
chrysene	ND	ND	1.59
C-1 chrysenes	ND	ND	1.59
C-2 chrysenes	ND	ND	1.59
C-3 chrysenes	ND	ND	1.59
C-4 chrysenes	ND	ND	1.59
benzo-b-fluoranthene	ND	ND	3.29
benzo-k-fluoranthene	ND	ND	3.00
benzo-e-pyrene	ND	ND	4.89
benzo-a-pyrene	ND	ND	3.03
perylene	ND	ND	5.39
indeno-123-cd-pyrene	ND	ND	8.74
dibenzo-a,h-anthracene	ND	ND	7.16
benzo-g,h,i-perylene	ND	ND	5.66
Total PAHs (ng)	**98.04**	**121.62**	

c) Hatch rates

While an ANOVA showed the accumulated temperature units (ATUs) to midhatch were not statistically different among treatments (p-value = 0.935, power = 0.05), we observed some trends in hatch times (Figure 3.11). In the "A" treatment scheme, the 500-ppm group took more ATUs to hatch than the controls and the 2500-ppm group took more ATUs to hatch than the 500-ppm group. This is consistent with the findings of the Chronic BY1999 experiment. We observed the same trend in the "B" treatment scheme, which is the opposite of what we observed in the Chronic BY2000 experiment. The "C" treatment scheme, similar to the acute assays at fertilization, took more ATUs to hatch than the controls and both the "A" and "B" treatment schemes.

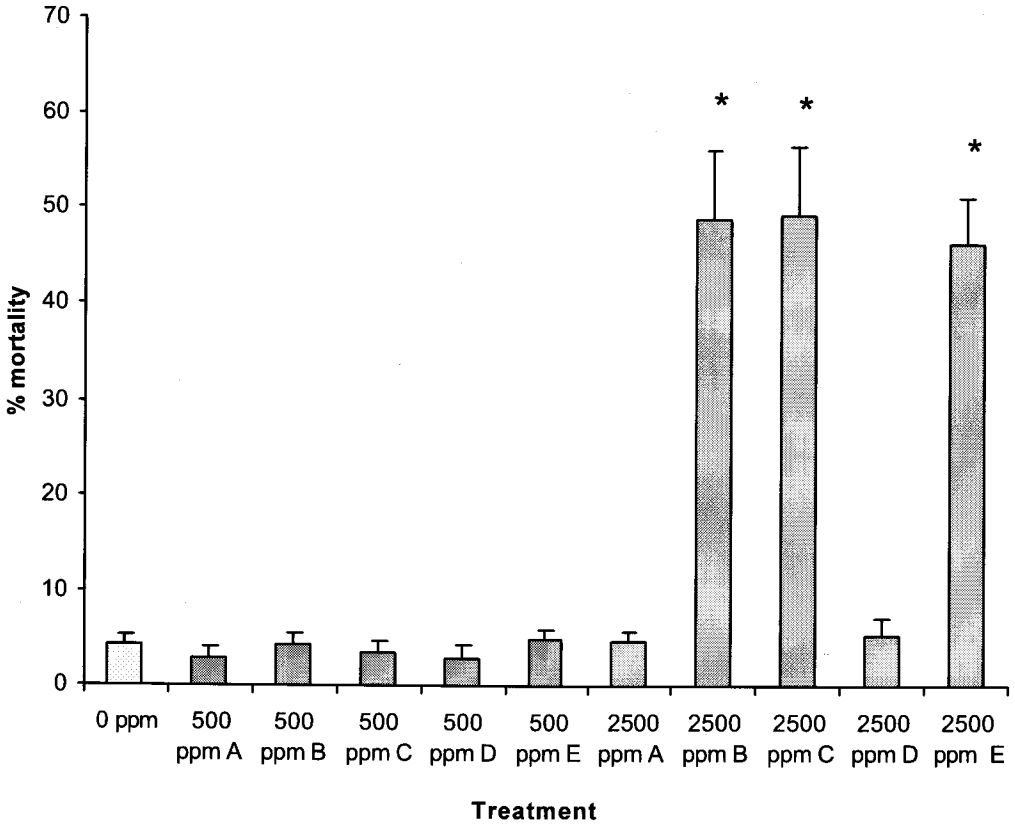
Figure 3.11. BY01 Accumulated Temperature Units to Hatch. Error bars are standard errors.



d) Fertilization and Mortality rates

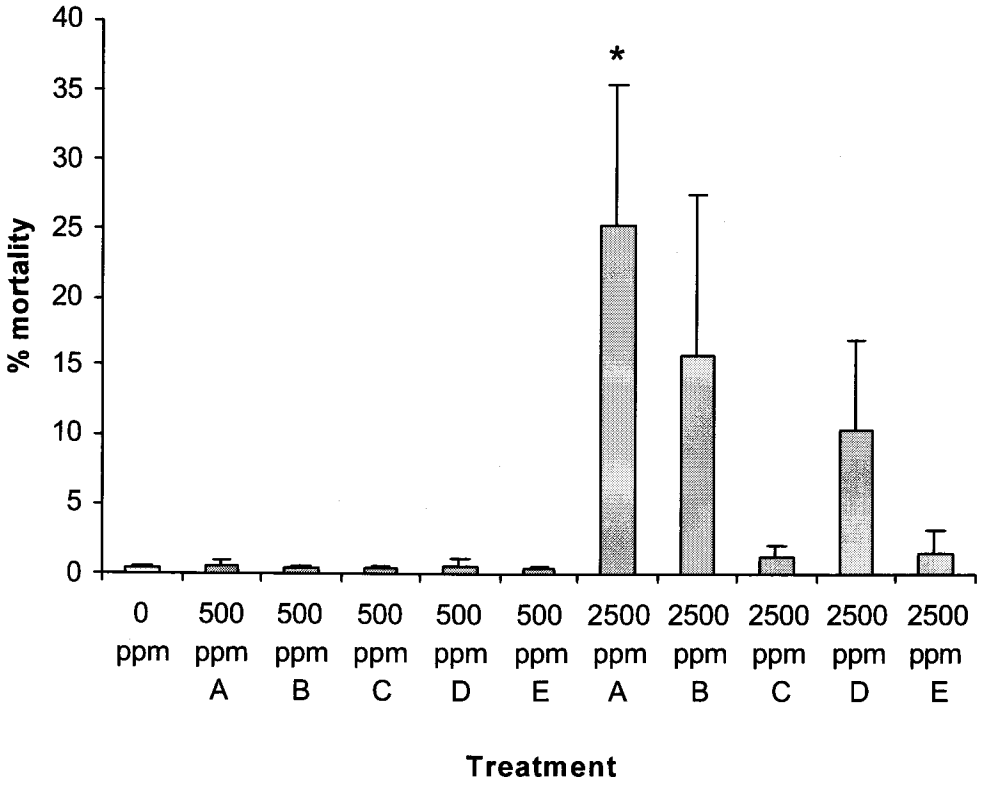
One trend was clear; exposure to 2,500 ppm during fertilization and/or during hatch was harmful to the fish. Prehatch mortality, eggs that were never fertilized or embryos that died before hatching, was high (nearly 50%) for the three exposure treatments that had eggs fertilized in 2,500 ppm, whereas all others had only around 5% mortalities (Figure 3.12).

Figure 3.12. Prehatch Mortalities. Percent of eggs that died before hatching. Error bars are standard errors. Asterisks denote significant difference from 0 ppm, $p<0.05$.



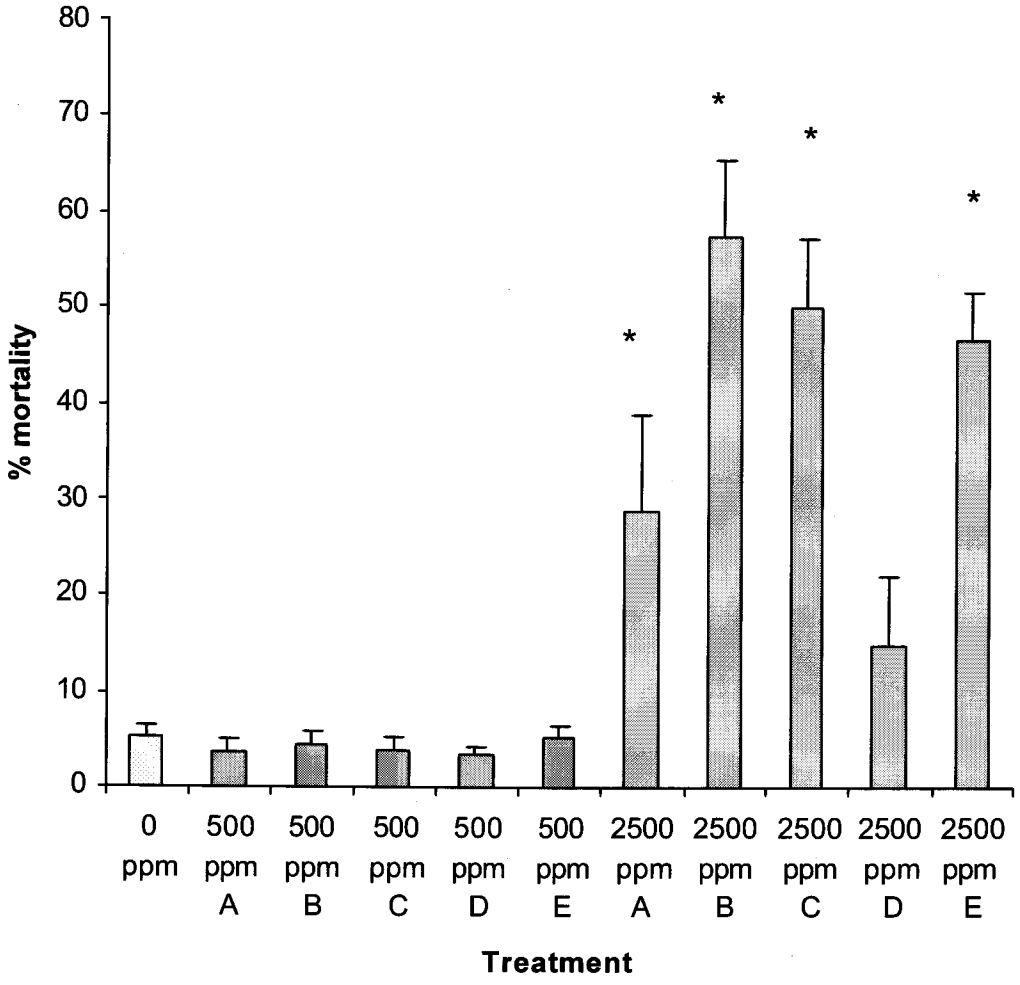
A few of the 2,500-ppm exposure treatments had posthatch mortalities (alevin that died between hatch and button-up) noticeably higher than the other treatments. The only treatment that was statistically different from the controls was the one in which eggs were fertilized and incubated in 0 ppm water for 24 hours and then switched to 2,500 ppm until button-up (Figure 3.13).

Figure 3.13. Posthatch Mortalities. Percent of alevin that died after hatching. Error bars are standard errors. Asterisks denotes a significant difference from 0 ppm, $p<0.05$.



Combining the prehatch and posthatch data showed that all embryos exposed to 2,500 ppm had higher mortalities than the 500-ppm treatments, especially those that were fertilized in 2,500 ppm. All but one exposure scheme using 2,500 ppm was statistically different from the controls (Figure 3.14). While the fish in 2,500 ppm D had lower cumulative mortality rates than 2,500 ppm A, a non parametric Student-Newman-Keuls Method of all pairwise comparisons showed there was no difference between the two treatments.

Figure 3.14. Cummulative Incubation Mortalities. Percent of eggs and alvin that died between fertilization and button-up. Error bars are standard errors. Asterisks denote significant difference from 0 ppm, $p<0.05$.



In contrast to the incubation mortalities, rearing mortalities looked similar to each other (ANOVA p-value = 0.12, power = 0.254), but the 0 ppm controls were lower than those incubated in TDS solutions (Table 3.10)

Table 3.10. Rearing Mortalities (% of fry that died) between May 24 and July 30, 2002.

	0 ppm	500 ppm	2,500 ppm	Solution Exposure Window
Tank 1	21	28	25	Fertilization to hatch
Tank 2	20	26	28	24 hrs post fertilization to button-up
Tank 3	18	23	18	Two min. post fertilization to button-up
Tank 4	16	21	25	Fertilization to button-up
Tank 5	26	26	26	Fertilization to two min. post fertilization
Mean	20	25	24	

e) Lengths and Weights

BY2001 Chronic exposed fish showed length and weight differences (ANOVA p-value = <0.001, power = 1.00) at clipping, May 24, 2002. Dunnett's Multiple Pairwise Comparison Tests showed that fish incubated in 500 ppm A and 500 ppm B were significantly heavier than the controls and 2500 ppm A and 2500 ppm B were significantly lighter than the controls (Figure 3.15). A similar pattern was seen in the lengths except, in addition to the fish exposed to 500 ppm and 2,500 ppm from fertilization to hatch (E), were significantly shorter than the controls (Figure 3.16). Roughly two months later, the lengths and weights on July 30, 2002 showed significant differences (Kruskal-Wallis ANOVAs, p <0.001 for both) but did not have a recognizable trend. There were a few treatments that had fish that were longer (500 ppm A and 2500 ppm C) and heavier (2500 ppm C, 500 ppm A, and 500 ppm C) than the 0 ppm control fish while one treatment was shorter and lighter (500 ppm D), as seen in Figures 3.17 and 3.18.

Figure 3.15. Broodyear 2001 Weights at Clipping on May 24, 2002. Error bars are standard errors. Asterisks denote significant difference from the 0 ppm controls, $p<0.05$.

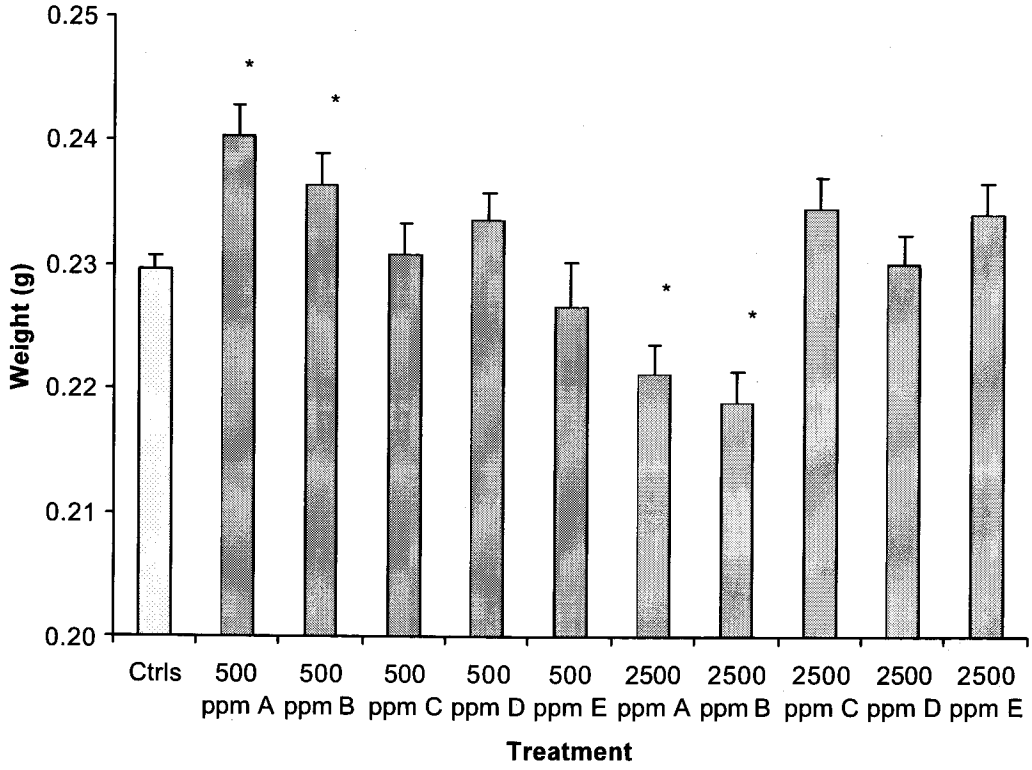


Figure 3.16. Broodyear 2001 Lengths at Clipping on May 24, 2002. Error bars are standard errors. Asterisks denote significant difference from the 0 ppm controls, $p<0.05$.

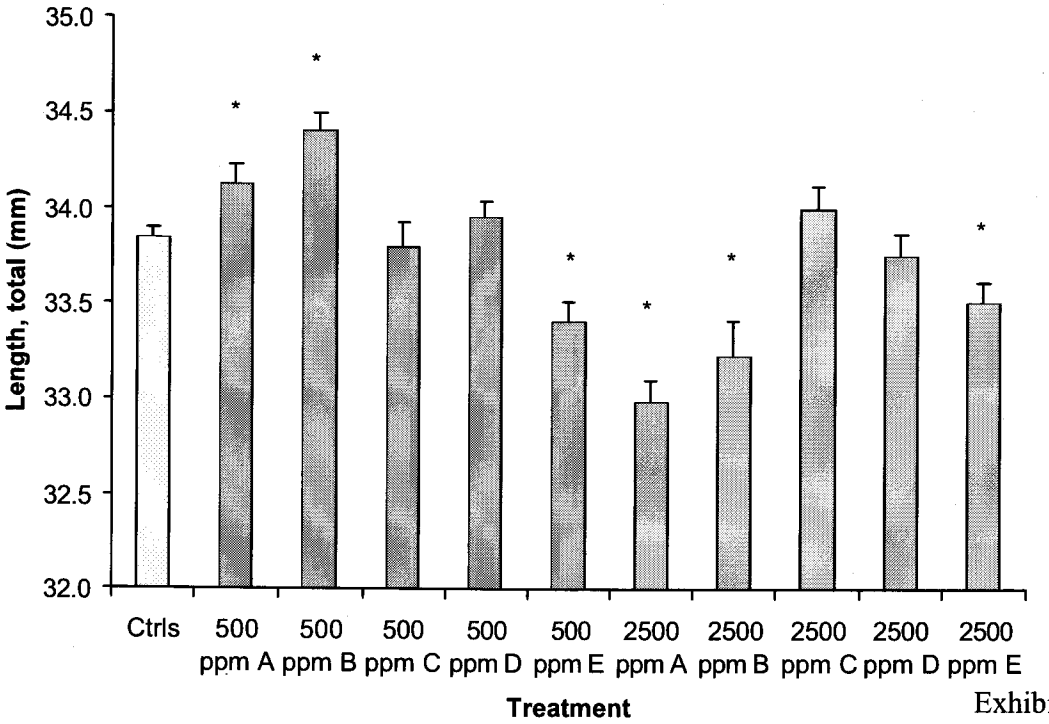


Figure 3.17. Broodyear 2001 Lengths on July 30, 2002. Error bars are standard errors. Asterisks denote significant difference from the 0 ppm controls, $p<0.05$.

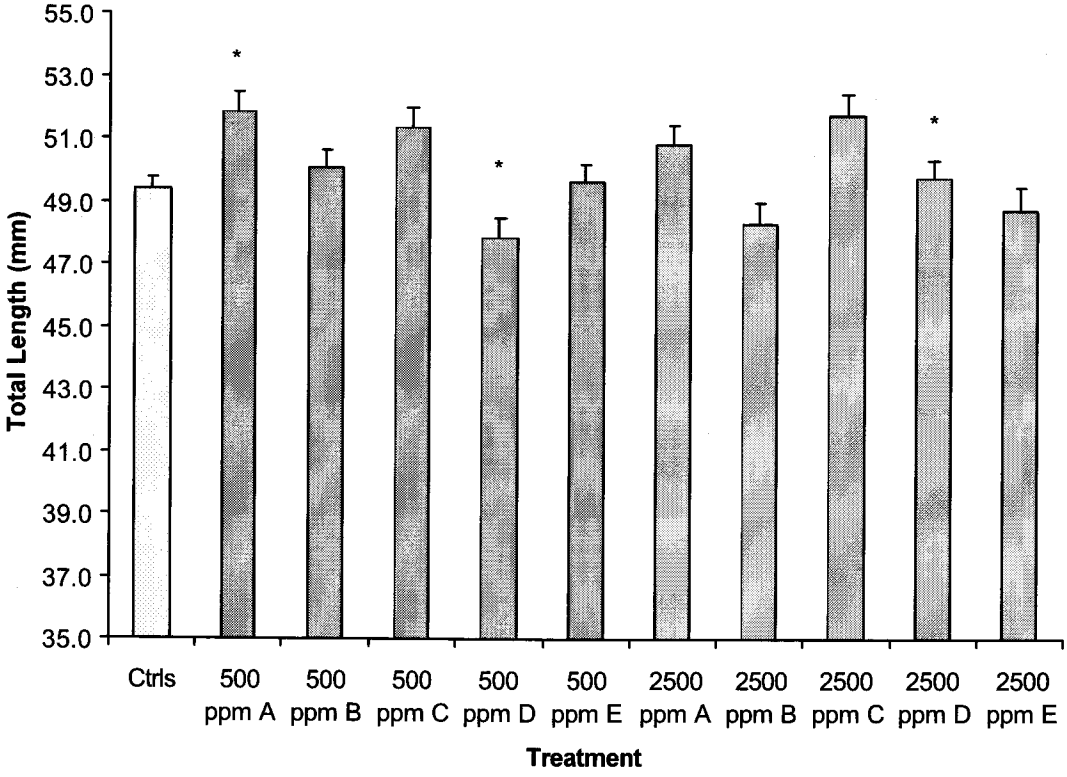
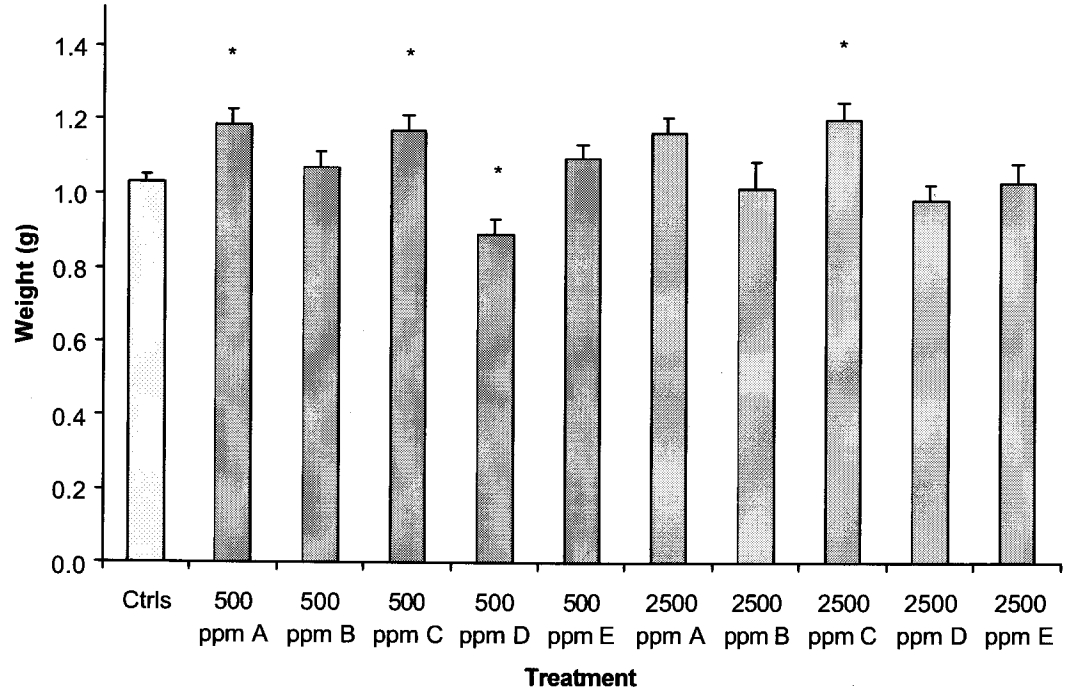


Figure 3.18. Broodyear 2001 Weights on July 30, 2002. Error bars are standard errors. Asterisks denote significant difference from the 0 ppm controls, $p<0.05$.



f) Histopathology

The Pathology Lab had one noteworthy observation; all fish had fluid accumulation in the cranial cavity. No other histological anomalies were observed (pers. comm., Ted Meyers, ADF&G Pathology Lab).

4. Discussion

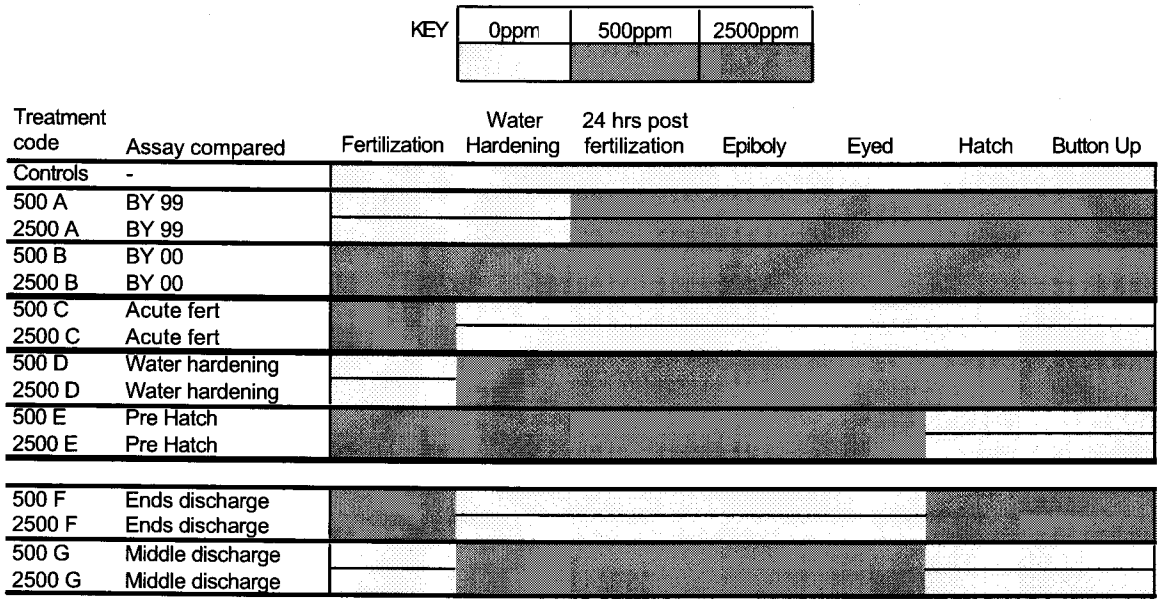
There were no water quality problems associated with these exposures in BY01. The maximum ammonia levels measured in BY01 were only 19% of the target level, roughly a third of the BY00, the next best year. This can be attributed to the consistent flushing of solutions by the peristaltic pump system. Based on samples taken during the winter of this broodyear and in the previous spring and summer, we conclude that hydrocarbons were not likely a factor in this experiment or previous ones.

BY01 had different hatch rate patterns compared to BY99 and 2000. BY01 was the first year that hatch rates were not statistically different among treatments. It is not totally clear to us what may have caused the differences in hatch timing in BY 1999 and 2000.

Histology does not appear to be affected by TDS exposure. Since all fish submitted for histological exam, 2500 ppm-exposed and controls, exhibited the same cranial swelling, Ted Meyers (ADF&G Pathology Lab) concluded that the condition was not due to TDS exposure but an artifact of the fixative. He also stated that if the TDS exposures are causing developmental aberrations during organogenesis prior to hatching, it is likely these changes will affect organ function rather than tissue architecture and therefore would not be discernable by histological examination.

Mortality rates in the BY01 Chronic experiment showed three obvious trends. First, 500 ppm showed no major effect. Second, fish exposed to 2500 ppm at fertilization had large mortality rates. Third, fish exposed to 2500 ppm during and after hatch had large mortality rates. We did not test two other treatment regimes that could have shed light on effects of TDS. The first is exposing fish during fertilization to 24 hours post fertilization and from hatch to button-up (F), and the second is exposing fish from after fertilization to before hatch (G) (Figure 3.19). It is clear from our results that exposure of high levels of TDS during fertilization and hatch is harmful to juvenile coho salmon. What remains unclear is what the effect would be of exposing juvenile coho salmon to high levels of TDS only in between those developmental stages.

Figure 3.19. Useful treatments missing from BY01 experiment. See Treatment codes "F" and "G" at the bottom.



Lengths and weights for fish exposed to 2500 ppm for the longest duration (Treatments A, B, D, E) tended to be shorter and lighter at the time of button-up (emergence). That trend was not discernable in the measurements taken two months later.

D. Chronic Assays Summary

It is important to emphasize that these results only apply to coho salmon from Macaulay Hatchery broodstock exposed to the simulated TDS mixture described above. Extensions to other populations of coho salmon may or may not apply. Drawing conclusions based on this data for other species is even less likely to apply. Applying this work to situations with different TDS mixtures, especially differing ratios of ions, additions or deletions of ions, and additional toxicants like heavy metals, is even more problematic. It is likely that other species will be adversely affected by TDS, especially during fertilization, but it is not possible to extrapolate our results to predict the effects at a given concentration.

The most notable effects we saw were related to mortality rates. Higher TDS concentrations at fertilization were related to higher pre-hatch mortality rates. Higher TDS concentrations at hatch were related to higher post-hatch mortality rates.

Results for time to hatch were varied. It is plausible that the earlier times to hatch for higher TDS exposed fish seen in Broodyear 2000 is the true effect. The opposite trend, in Broodyear 1999 was likely caused by ammonia toxicity instead of TDS exposure. We did not see a cohesive logical pattern for times to hatch in Broodyear 2001. High variance in certain treatments may have masked effects of TDS. The low power of the statistical test precludes drawing firm conclusions for that broodyear.

In all broodyears, for fish that were exposed to higher concentrations of TDS, lengths and weights at the time of button-up (emergence) tended to be smaller. In all broodyears, the differences were not persistent. We conclude that in an environment where demands for juvenile salmon (competition for food, predator avoidance and physiological challenges) are low, TDS-related size variation may not be important, but in more challenging environments, size differences may lead to lower survival rates in fish exposed to higher concentrations of TDS during incubation.

Based on our results, site-specific tests may be the best method to use to set limits for TDS in issuing discharge permits. Such tests could include short tem bioassays at critical stages, such as fertilization or hatch. Other long-term assays could be employed if deemed necessary for understanding effects to critical populations. The results described here are relevant only directly to salmonid populations. The effects of TDS on other organisms in an ecosystem in question may also play an important role in the growth and survival of salmonids.

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