EFFECTS OF THE AQUATIC HERBICIDE *CASCADE*® ON SURVIVAL OF SALMON AND STEELHEAD SMOLTS DURING SEAWATER TRANSITION

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ABSTRACT

Determining sublethal effects of pesticide exposure on anadromous salmonids as they transition from freshwater to seawater is critically important in the Pacific Northwest due to the presence of commercially and culturally valuable salmon and steelhead populations. The widely used aquatic herbicide endothall has relatively low toxicity to salmonids following initial exposure (LC50 of 32-230 ppm a.e.), but effects of low application concentrations on osmoregulatory performance of seagoing juveniles have not been adequately investigated. Previous studies relied on small sample sizes, inappropriate life-stages, static exposure and seawater systems, and insufficient challenge durations, generating contradicting results. To resolve uncertainty about endothall effects on anadromous salmonids, coho (*Oncorhynchus kisutch*), Chinook (*O. tshawytscha*), and steelhead (*O. mykiss*) were subjected to a ten-day seawater challenge following acute exposure to *Cascade*® (endothall dipotassium salt formulation) in a flow-through system. Acute exposure ranged from 0 to 12 ppm acid equivalent (a.e.) endothall for 96 h. The seawater challenge yielded mean survival rates of 82% (n=225), 84% (n=133), 90% (n=73) and 59% (n=147) for 0, 3-5, 6-8, and 9-12 ppm a.e. exposure groups, respectively. Steelhead exhibited a statistically lower survival rate at exposures >9 ppm a.e, relative to coho and Chinook. Surviving fish did not experience significant changes in osmoregulatory performance compared with control fish, as revealed by plasma sodium analysis. Lowest observable effect concentrations were 9 ppm a.e. for steelhead and 12 ppm a.e. for coho and Chinook, indicating a lower effect threshold compared with results reported from previous acute toxicity studies, but a higher threshold compared with results from some previous seawater challenge experiments. Our findings emphasize the importance of conducting carefully designed seawater challenge experiments before defining chemical toxicity levels in areas with anadromous fish.

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INTRODUCTION

Anadromous fish species rely on freshwater habitats for reproduction and juvenile rearing, and saltwater habitats for subadult growth and adult maturation. The transition between freshwater and saltwater environments, though achievable, is a precarious event, particularly for juveniles entering the ocean at a relatively small size, and marine survival rates are typically low due to a variety of sources of mortality (Parker 1971; Mathews and Buckley 1976; Walters et al 1978; Healey 1982). During seawater transition, salmon and steelhead must shift from a freshwater or hypotonic environment to a hypertonic environment. This transition requires adaptation of the branchial epithelia to accommodate the change in osmotic and ionic gradients in order to maintain physiological homeostasis. In freshwater, fish must allow the inflow of ions and osmotic water loss, whereas in a marine environment there must be an outflow of ions and osmotic uptake of water. A reduced osmoregulatory capacity caused by chemical exposure would impair the physiological transition from freshwater to saltwater, which could be detrimental to the ability of anadromous fish to complete their life-cycle.

Pacific Salmon and steelhead are varieties of anadromous salmonids common throughout the Pacific Rim. They hatch in inland and coastal streams, rear in freshwater for one to four years, migrate to the ocean, and return as mature adults one to four years later, often spawning within just a few meters of their natal rearing habitat. Depressed abundance of anadromous salmonids in the Pacific Northwest and Northern California has led to extensive efforts to

research causes of declines and prevent future adverse effects of anthropogenic changes to freshwater habitat conditions. There are several regulatory mechanisms in place to protect salmon and steelhead, the most prominent of which is the U.S. Endangered Species Act (ESA). Currently, eleven populations of steelhead trout, nine populations of Chinook salmon, two populations of chum salmon, four populations of coho salmon, and two populations of Sockeye salmon are listed as either threatened or endangered under the ESA.

ESA listing status and cultural and economic importance of Pacific salmon and steelhead has also affected other regulatory policies, including the National Pollutant Discharge Elimination System (NPDES) permitting process implemented by state government agencies on behalf of the U.S. Environmental Protection Agency (EPA) under the Clean Water Act. Chemicals discharged to natural water bodies that may adversely affect Pacific salmon and steelhead are often reviewed with additional scrutiny. Moreover, because salmon and steelhead are anadromous, their two-phase life-cycle is considered when determining toxicity and subsequent regulated discharge levels.

In Washington State, the Washington Department of Ecology (WDOE) is chiefly responsible for issuing NPDES permits, but they often consult with other agencies, such as the Washington Department of Fish and Wildlife (WDFW), when determining allowable discharge levels for certain chemicals. In the case of aquatic herbicides and pesticides, state agencies are allowed to be more restrictive, but not less restrictive than levels approved by the Federal government.

The aquatic herbicide endothall (7-oxabicyclo[2.2.1]heptaine-2,3-dicarboxylic acid) is commonly used to control aquatic plant species because of its high efficacy at low concentrations (Carlson et al 1978; Westerdahl and Getsinger, 1998). Two derivatives of endothall are commercially available, which include the mono (N,N-dimethylalkylamine) salt and the inorganic salt (dipotassium or disodium). The 40.3% dipotassium salt formulation is manufactured under the label *Cascade*® by United Phosphorus, Inc (King of Prussia, PA). In Washington State, *Cascade*® has been permitted for use by WDOE for aquatic weed control, including Eurasian water milfoil *Myriophyllum spicatum*, in irrigation water conveyances. The State of Washington Department of Ecology has concluded endothall's risk to be relatively low compared with other alternatives (Halter 1980) and it is unlikely to cause adverse chronic impact to any segment of the biota (WDOE *Herbicide Risk Assessment for the Aquatic Plant Management Final Supplemental Environmental Impact Statement* (DOE EIS)). Currently, application limits for *Cascade*® are under review by the Washington State's NPDES process.

The dipotassium endothall salt formulation has low acute and chronic toxicity effects toward fish and other animals compared with levels needed to effectively treat aquatic weeds. For example, the highest level approved by the EPA and recommended by the manufacturer for weed control is 5 parts per million (ppm) acid equivalent (a.e.) endothall, while the lethal concentration, 50% (LC_{50}) for various salmonids is found to be greater than 20-to-40 times the maximum recommended application rate. In previous studies acute LC_{50} values

ranged from 32-230 ppm a.e. for rainbow trout (Mayer and Ellersieck, 1986; Bettencourt 1993), >100 ppm a.e. for coho salmon (Johnston and Finley, 1980; Mayer and Ellersieck, 1986), and 23 ppm a.e. for Chinook salmon (Pennwalt Corporation 1986, as reported in WDOE EIS). The no observable effect concentration (NOEC) for rainbow trout ranged between 41-51 ppm a.e. (DOE EIS), approximately ten times more than the application label permits. A chronic (14 day) study by Liguori et al. (1983) found the LC_{50} for Chinook at 88 ppm a.e.

These freshwater studies revealed a relatively low acute toxicity of endothall to anadromous salmonids. However, there is evidence that smolts (emigrating juveniles) exposed to low endothall concentrations may experience reduced seawater adaptability (Bouck and Johnson 1979; Liguori et al. 1983) suggesting the possibility of sublethal effects of endothal exposure that do not manifest themselves until juveniles transition to a marine environment. A seawater challenge experiment is a sensitive and useful determinant of sublethal effects of toxicant exposure for anadromous salmonids, which takes into account their two-phase life cycle (Bouck and Johnson, 1979; Ligouri et al 1983; Serdar and Johnson 1993). Histopathological analysis revealed mild gill inflammation at 10 ppm a.e. and researchers suggested that reduced seawater survival was due to endothall acting as an irritant to gill epithelial cells and therefore caused improper osmoregulatory function (Ligouri et al 1983). However, when a 24-hour seawater challenge was carried out following endothall exposures as high as 10 ppm a.e., fish did not experience mortality or abnormal osmoregulatory capacity as measured by plasma sodium analysis (Serdar and Johnson, 1996). Published

reports on the sublethal effects of endothall exposure vary and often contradict, highlighting the influence of study design on experimental outcome.

Experimental design discrepancies and substantial differences between results of previous studies led to considerable uncertainty regarding effects of endothall on anadromous salmonids, particularly as the fish transition into seawater. Differences between previous studies include sample sizes, species and life-stages used, fish density, static or flow-through chemical exposure and seawater challenge systems, length of endothall exposure and seawater challenge, chemical formulations used during exposure, etc. This makes it difficult to adequately conclude whether *Cascade*® has an effect on the ability of anadromous salmonids to transition from freshwater to seawater. Lacking sufficient data, WDOE relied on the lowest observed effect level (3 ppm a.e.) to construct conservative threshold concentrations of 1.0 ppm a.e. in the spring and 2.5 ppm a.e. in the summer/fall for the discharge of *Cascade*® where treated water enters natural water bodies (NPDES Permit Modification, March 17, 2010).

To resolve data uncertainties, a rigorous seawater challenge study was needed to assess the ability of Pacific salmon and steelhead to adapt to a marine environment following exposure to *Cascade*®. This study takes into account:

1) appropriate test species;
2) proper life-stages;
3) adequate numbers of fish;
4) chemical exposure duration equivalent to or greater than what would be expected during field application;

5) a flow-through system to allow proper circulation and flow
6) water temperature conditions equivalent to or higher than would be expected during salmonid smolt exposure following field application;
7) a seawater challenge duration sufficient to capture delayed mortality following seawater entry; and,
8) meticulous attention towards the maintenance of proper water quality parameters to ensure optimal husbandry conditions.

METHODS

Chemical Handling

Proper health and safety precautions were taken when handling, preparing, and disposing of *Cascade*®. Direct exposure to light was as also avoided during storage and application of *Cascade*®. Appropriate permits were obtained and approval for use and disposal was granted from the City of Troutdale.

Fish Husbandry

Strict protocols were established for careful handling of chemical compounds, acquisition and transport of fish, fish health, lab procedures, and data collection in accordance with the *Guidelines for the Use of Fish in Research* (American Fisheries Society) and *Good Laboratory Practice Standards* (U.S. EPA).

Juvenile salmon were obtained from Washington Department of Fish and Wildlife hatchery programs in the lower Columbia Basin. Coho salmon, steelhead trout and fall Chinook salmon were collected from the Lewis River, Merwin, and Washougal Hatcheries, respectively, and transported to the Fisheries Technology laboratory facility at Mt. Hood Community College (MHCC) in Gresham, Oregon during the first week of May, 2011. Salmon were transported to MHCC by truck and appropriate precautions were taken to reduce stress and injury. Following transport, a fish pathologist confirmed animal health and fish were allowed to acclimate in 350-gallon holding tanks for two weeks prior to endothall exposure. Recirculated well-water was UV-treated and passed through ammonia-fixing bacteria (Aquabac-T, Argent Labs, Redmond, WA) and polypropylene biomedia to minimize ammonia and nitrite levels. Fish were exposed to a natural light photoperiod of 16 h light and 8 h dark and maintained at 14-15°C. Oregon Moist Pellets (Moore Clark Co., LaConner, WA) were administered *ad libitum*, and feeding stopped 24 hours prior to transfer to testing system and fish were not fed for the duration of the study. Prior to endothall exposure or the seawater challenge, aquaria and tanks were sanitized with Wescodyne (West Chemical Co, NY) and thoroughly rinsed prior to experimental setup and between experimental trials. Fish were monitored daily for mortalities, and behavioral and anatomical abnormalities.

Water Quality

The optimum range for water quality parameters – total dissolved gasses (> 90%), dissolved oxygen (> 70%), pH (6.7 - 8.5), temperature (between 14-16°C), total ammonia (NH_4^+, < 0.5 ppm), un-ionized ammonia (NH_3, ≤ 0.03 mg/L), nitrate/nitrite (≤ 0.55), water flow (13.25 L/min; acute toxicity test), and salinity (30 ppt; seawater challenge) – were monitored and maintained daily. Reagents used were compatible for both freshwater and saltwater use; pH (PrimaLine, ELOS, Verona, Italy), ammonia (API, Chalfont, PA), and nitrogen (API, Chalfont, PA). Within the seawater challenge system, ammonia and nitrate/nitrite levels were controlled by the use of Proline® ammonia remover (Aquatic Ecosystems, Inc, Apopka, FL) and carbon filtration, according to the manufacturers' recommendations.

Chemical Delivery

The aquatic herbicide *Cascade*® (United Phosphorus, Inc, King of Prussia, PA) was obtained from the Washington State Water Resources Association (Olympia, WA). *Cascade*®contains 40.3% (wt/wt) dipotassium salt of endothall and 28.6% (wt/wt) or 36% (wt/vol) dicarboxylic a.e. (361 mg acid per milliliter). The concentrated solution of endothall was mixed with dilution water in a 2 L Mariotte bottle prior to delivery to the test aquaria. In order to maintain sustained release of the herbicide, a chemical delivery system consisting of fluid controlled dispensing pumps (Fluid Metering, Inc, Syosset, NY) and chemical mixing chambers were utilized upstream of the glass test aquaria (635 L). Adequate

chemical mixing was achieved and confirmed by the chemical dye fluorescein (uranine) and time-lapse photography. The Mariotte bottle, or the chemical reservoir, was positioned above the aquaria, which provided adequate head pressure supplying the dispensing pumps. The herbicide was allowed to flow from the FMI pump into the mixing chamber at 1 mL/13.25 L/min. The Mariotte bottle was also covered to prevent the herbicides' exposure to light. The entire exposure system was recalibrated prior to each experimental trial and tested at multiple intervals during each trial to ensure proper concentrations of endothall within each aquarium.

96-Hour Acute Toxicity Test

A flow-through system was used and water (well-sourced) flowed at a rate of 13.25 L/min, regulated by in-line digital flowmeters (Aquatic Ecosystems, Inc, Apopka, FL), allowing for complete water replacement in 48 min. Temperature was maintained using an in-line circulation heating system (Condex Wattco Inc., Lachine, Quebec), and continually monitored using HOBO electronic data logging system and software (Onset, Pocasset, MA).

Steelhead trout, coho salmon and/or fall Chinook smolts were transferred from the 350-gallon holding tanks to an aquarium. Following 24 hours of acclimation, endothall exposure began and temperature was incrementally increased 2°C per hour until the desired temperature of 20°C was achieved. Temperature was maintained within ±0.5°C for the duration of the 96-hour exposure. In total, each aquarium contained 120 smolts, 30 of each species, for

a total of 4 experiments. Water samples were collected at 24 and 72 hours from each treatment aquarium to be tested for the nitrogen derivative of the parent compound using a gas chromatograph/mass spectrometric (GC/MS) method (U.S. EPA Method 548.1; Anatek Labs, Moscow ID). Water samples were set to pH 2.0 with HCl upon collection. Treatment groups consisted of 0, 3.5, 5, 7.5, and 10 ppm. Actual endothall concentrations as measured by GC/MS for each trial at both 24 and 72 hours are provided in detail in Table 1 of Appendix A.

Seawater Challenge

After 96 hours of endothall exposure, the chemical delivery system was turned off and the water temperature was decreased by 2°C per hour. Fish were maintained at well-water temperature (14-15 C) for 24 hours and then transferred to 40-gallon circular tanks in a closed flow-through system containing fresh well-water, where they were held for 24 hours before water was gradually replaced with seawater using H2Ocean Magnesium Pro Plus (D-D The Aquarium Solution, Ltd., Scottsdale, AZ), similar to the methods of Clarke and Blackburn (1977) and Serdar and Johnson (1996). Salinity was brought to 8 ppt following 24 hours acclimation, and increased to 20 ppt at 8 hours and 30 ppt 24 hours post-salt introduction. For trial 4 (only trial used for steelhead plasma sodium analysis), salinity was brought up gradually – 6 ppt at 17 h, 12 ppt at 32 h, 18 ppt at 2.5 d, held at 24 ppt for 3 d (Days 4-6), 28 ppt at 7 d, and 30 ppt for Days 8-10. Water temperature was maintained at 14-15°C, as previously mentioned, using a Teco SeaChill chiller (Aquatic Ecosystems, Inc, Apopka, FL). The ten-day seawater

challenge began 24 hours after first introduction to seawater. Surviving fish at the end of the seawater challenge were either given a lethal dose of MS-222 prior to disposal or euthanized by stunning for blood sample collection.

Plasma Sodium Analysis

On day ten, blood was collected from the caudal artery (fall Chinook) or by cardiac puncture (steelhead, coho) using lithium-heparin (MP Biomedicals, Solon, OH) coated needles and collection tubes. Blood was pooled from 2-3 fish (steelhead), 4-5 fish (coho), and 7-10 fish (fall Chinook); per treatment, in duplicate or triplicate. Samples were immediately placed on ice and transported to the Core Laboratory at Oregon Health & Science University (Portland, OR) for plasma sodium analysis. Plasma sodium levels were determined by a SYNCHRON® system (Beckman Coulter, Brea, CA) utilizing indirect potentiometry. Data analysis of plasma sodium concentrations involved comparing the mean of all replicates between treatment groups (student's t-test).

Contaminant Analysis

Effort was taken to ensure fish were not exposed to contaminants or toxins/toxicants through the water supply. Well-water samples were taken and test results met all requirements of NELAC (National Environmental Laboratory Accreditation Conference, U.S. EPA); analysis for various metals and suspended solids were found below detectable levels (Pyxis Labs, Portland OR). In addition, threaded Schedule 80 PVC piping was installed for all in-flow plumbing to avoid

leaching of adhesive-related contaminants into the testing system. Water samples were taken from each test aquaria prior to fish introduction to test for the detection of PVC-related contaminants (Anatek Labs, Moscow, ID). Vinyl chloride, tetrahydrofuran, and methyl ethyl ketone were below detection at the ppb level.

Survival Data Analysis

Generalized Linear Mixed Models (GLMM) were fit to the data to evaluate how different levels of endothall affected overall survival of each species. GLMMs are similar to General Linear Models (GLMs) (e.g. logistic regression), but have a number of distinct advantages over GLMs and are strongly recommended in ecological studies (Bolker et al. 2007). Common reasons for using GLMMs over GLMs include: (1) accounting for possible relatedness between observations in experimental units that may be similar; (2) keeping the model parsimonious; and (3) extending inference from the observations used in the dataset to the population in general.

GLMMs can be decomposed into two components: the fixed and random effects. Fixed effects are covariates or treatments whose interest lies in the specific effect of that variable on the response (i.e. the effect of the *Cascade*® on survival). Random effects are variables whose interest lies in the variation among them rather than the specific effect of each of them on the response. For example, describing how survival varied between trials was of less interest compared to describing the variation in conditions that fish experienced within a

single trial. Single or multiple random effects can be included in GLMMs. Potential variation in tanks from different trials in the study were modeled by including them as nested random effects in all competing models (e.g. Preisler 1989). By doing so, statistical models were expected to better explain how different levels of *Cascade*® affect survival on the three different species of fish by accounting for small differences in tanks that may have varied from trial to trial. This was important because, although this was a controlled lab study, perfect control of every variable that may affect survival is not possible. Specific examples include chemical exposure levels, flow rates, and changes in fish size between trials. The GLMM approach allowed us to account for these small differences, which may otherwise bias statistical results.

When modeling the fixed effect structure, *Cascade*® exposure was treated as a categorical variable defined by four groups: control (0 ppm a.e.), 3-5 ppm a.e., 6-8 ppm a.e. and 9-12 ppm a.e. endothall. These four groups can generally be thought of as control, low, medium and high respectively. This approach was preferable to treating endothall as a continuous covariate because it facilitated testing whether there were significantly different rates of survival between different levels of *Cascade*® exposure[1]. It should be acknowledged that many different groupings could have been defined. The four groups chosen for this study ensured that each species was represented within each category of *Cascade*® exposure level.

[1] Treating endothall as a continuous covariate would only indicate whether the trend of endothall on the log odds of survival was significant.

Three different competing models with different fixed effect structures were fit to the dataset. The best fitting model was chosen according to AIC selection criteria (Burnham and Anderson 2002). In general all models have the form

$$
\begin{aligned}
&Y_{i,j,k,s} \sim \text{Bin}(1, p_{i,j,k,s}) \\
&\text{logit}(p_{i,j,k,s}) = f(\beta_{i,j,}; \alpha_s) + a_i \times b_j \\
&a_i \sim \text{N}(0, \sigma_a^2) \\
&b_j \sim \text{N}(0, \sigma_b^2)
\end{aligned}
$$

$Y_{i,j,k,s}$ is distributed binomially and equals 1 if a fish *k* of species *s* from tank *j* in trial *i* is alive at the end of the study and equals 0 if the fish died during the ten days. *p* is the probability that of survival to the end of the study. logit(*p*) = log(*p* / (1-*p*)) and is interpreted as the log odds of survival. $f(\beta_{i,j}; \alpha_s)$ is the functional form of the model that depends on the intercepts describing the mean survival for the *Cascade*® exposure level applied to tank *j* in trial *i* ($\beta_{i,j}$) and species *s* (α_s). This function varies in each of the three competing models. a_i x b_j is the nested random effect modeled as intercepts between tank *j* and trial *i.* Each intercept is assumed to be normally distributed with mean 0 and respective variance σ_a^2 and σ_b^2.

Table 1 displays the functional forms of $f(\beta_{i,j}; \alpha_s)$ in each of the competing models. Model 1 assumed that the log odds of survival depends only on the level of *Cascade*® and there is no difference between species. Model 2 assumed the log odds of survival depends on the additive effect of the level of *Cascade*®

and species. Model 3 is the "saturated model"[2] and assumed that the log odds of survival depends on the additive and multiplicative effect of the level of *Cascade*® and species. Note that Model 2 allowed testing whether survival differed amongst species and whether survival differed amongst levels of endothall, whereas Model 3 allowed testing whether survival differed amongst species for a specific level of *Cascade*® treatment.

The three competing models are fit using the R Statistical Platform (R Core Development Team 2011). Specifically the lme4 package (Bates 2011) is used to fit the GLMM models.

Table 1. Description of the six different functional forms of species and endothall used in the five competing models.

	f(Endothall ; α_k)
Model 1	$\alpha + \beta_{i,j}$
Model 2	$\alpha_s + \beta_{i,j}$
Model 3	$\alpha_s + \beta_{i,j} + (\alpha_s \times \beta_{i,j})$

[2] In the statistical nomenclature, the "saturated model" indicates that the number of parameters in the model equals the number of points being fit. That is, if there are 12 points of interest (3 species x 4 levels of endothall) then the model also fits 12 parameters. Model 2 and 3 are both able to predict 12 survival probabilities, but model 2 uses less parameters.

RESULTS

Survival

Patterns in survival indicate that *Cascade*® treatments between 0 and 8 ppm a.e. of endothall did not have an effect on survival of steelhead trout, coho salmon, or Chinook salmon during a ten day seawater challenge, but treatments between 9 and 12 ppm a.e. endothall reduced survival for all three species relative to other treatment groups. Survival reductions in the 9-12 ppm a.e. endothall treatment groups relative to control fish were 20%, 13%, and 48% for coho, Chinook, and steelhead, respectively. Patterns in survival were consistent during all trials with mortality beginning on Day-3 or 4 and subsiding by Day-8 or 9 of the seawater challenge (Figure 1).

AIC values for the three competing statistical models are presented in Table 2. Model 2, which included an additive effect of endothall and species on the log odds of survival was most strongly supported by the dataset according to AIC selection criteria. Statistical output from R provided by the lme4 package for this model is presented in Appendix B.

P-values for all pairwise comparisons between species and level of *Cascade*® treatment are provided in Table 3. Mean survival for steelhead was statistically different compared to coho and Chinook, but mean survival was not statistically different between coho and Chinook. Mean survival was significantly different for 9-12 ppm a.e. endothall treatments compared to all other levels, but

was not significantly different for control (0 ppm a.e.), 3-5 ppm a.e. and 6-8 ppm a.e. comparisons.

Modeled predictions of survival, which account for uncontrolled variability between experimental trials, for each species when treated with different levels of *Cascade*® are presented in Figure 2. Survival was always noticeably lower for steelhead compared to Chinook and coho. Survival was relatively constant for the control, 3-5 ppm a.e. and 6-8 ppm a.e. treatments and then declined for 9-12 ppm a.e. endothall treatment groups.

Figure 1. Daily survival rates of coho salmon, Chinook salmon and steelhead trout following exposure to *Cascade*® and subjection to a 10-day seawater challenge.

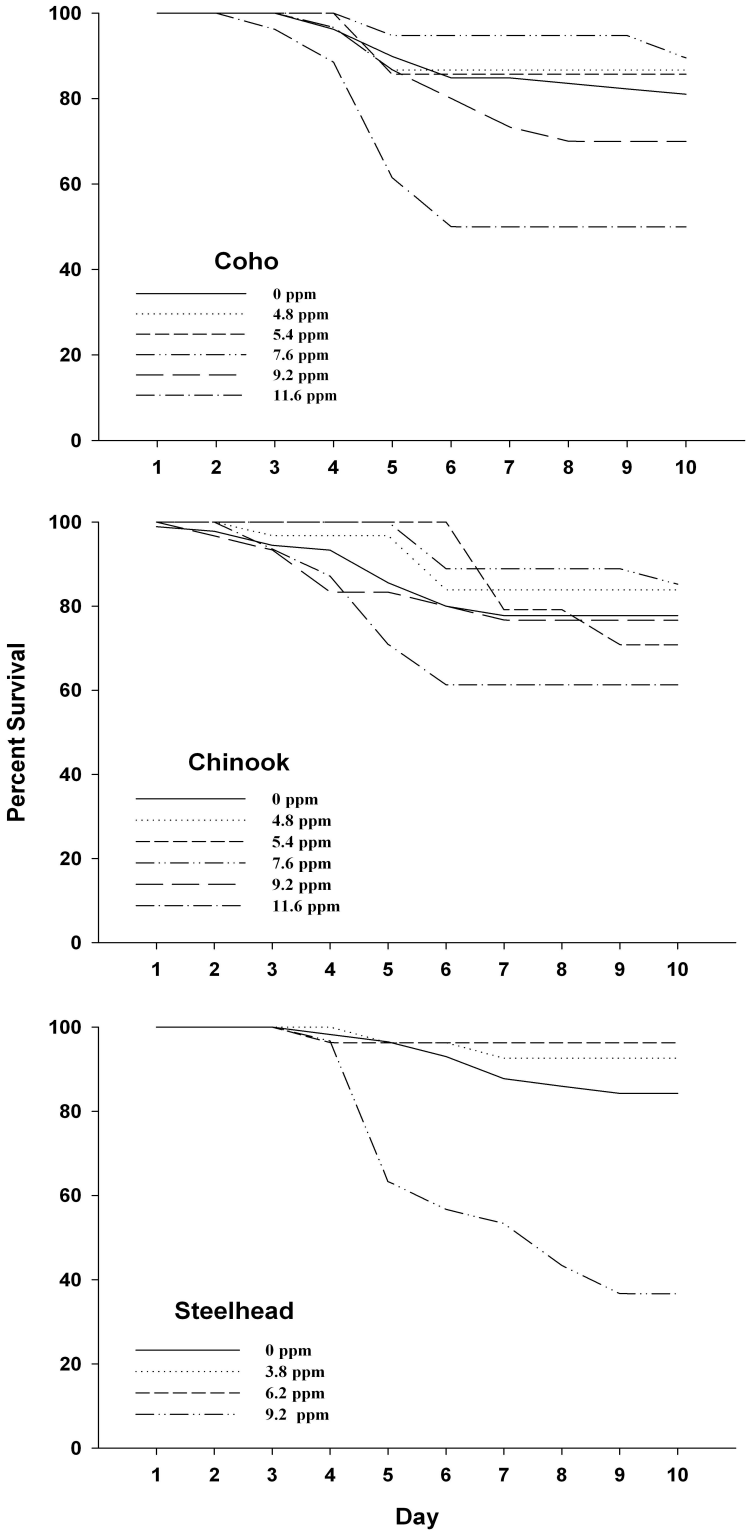


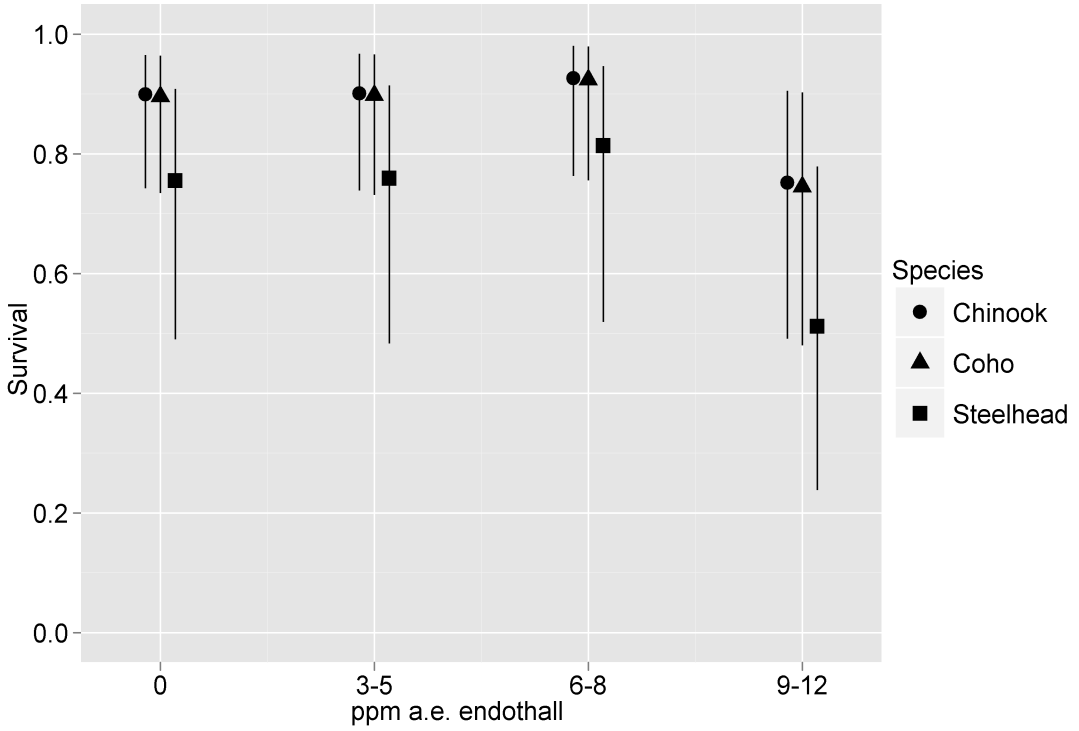
Table 2. AIC values for the six competing models.

	AIC
Model 2	575.5
Model 1	575.8
Model 3	581.7

Table 3. P-values for comparing whether survival was significantly different between species and levels of endothall from Model 2.

Species		Chinook	Coho	Steelhead	
	Chinook	-	0.87	< 0.01	
	Coho	-	-	< 0.01	
	Steelhead	-	-	-	
Endothall	(ppm a.e.)	0	3-5	6-8	9-12
	0	-	0.94	0.48	< 0.01
	3-5	-	-	0.51	< 0.01
	6-8	-	-	-	< 0.01
	9-12	-	-	-	-

Figure 2. Predicted survival probabilities for Chinook salmon, coho salmon, and steelhead trout following exposer to four different levels of *Cascade*® (expressed as endothall in ppm a.e.) and subjection to a 10-day seawater challenge.



Plasma Sodium Concentrations

Blood was collected from surviving fish on day-10 of the seawater challenge. Plasma sodium concentrations were not significantly different between treatment and control groups for coho, Chinook, and steelhead ($p > 0.05$, student's t-test; Table 4). Mean plasma sodium concentrations measured from coho (158 mmol/L) and fall Chinook (155 mmol/L) did not differ significantly from concentrations measured from fish exposed to freshwater (148 mmol/L). Mean plasma sodium concentrations for steelhead measured 188 mmol/L. All data, including raw data collected for each replicate within treatments, are provided in Table 3 of Appendix A.

Table 4. Summary plasma sodium data collected following seawater challenge trials.

Species	Dose (ppm a.e.)	Mean Plasma Sodium (mmol/L)	Mean Plasma Sodium between treatments (mmol/L)
Steelhead	0	196 ± 10.83	188 ± 8.3
	3.77	188 ± 7.29	
	6.17	179 ± 12.58	
Coho	0	156 ± 5.13	158 ± 2.9
	5.43	157 ± 4.25	
	7.58	161 ± 1.26	
Fall Chinook	0	152 ± 4.77	155 ± 5.4
	5.43	162 ± 3.54	
	7.58	153 ± 5.39	
Freshwater Only	--	148 ± 11.31	--

Blood was collected from fish in 14-15° C seawater on 09/01/11 (coho and fall Chinook) and 09/16/11 (steelhead). No significant difference in plasma sodium levels were observed between *Cascade*® treatments and controls, and values represent the mean plasma sodium levels of each treatment. The densities of the fish for the single trial for blood collection were 0.98, 0.39, and 0.08 g/L for steelhead, coho and fall Chinook, respectively.

DISCUSSION

The seawater challenge is a useful and sensitive assay that determines sublethal effects of chemical exposure on juvenile seagoing fish (Ligouri et al 1983; Serdar and Johnson 1993; Bouck and Johnson 1979), and it is often underrepresented in studies investigating toxic effects on anadromous species. This test identifies whether toxicant exposure may disturb the ability of fish to maintain internal equilibria of osmotic and ionic exchange by (a) directly affecting the physical structure of gill epithelia; (b) altering the smoltification process; and/or (c) evoking stress in the animal.

We investigated the effects of *Cascade*®, the dipotassium salt formulation of endothall on the ability of anadromous salmonids to transition to seawater. The relatively low toxicity of *Cascade*® to salmonids in freshwater have been well established; however, *Cascade*'s® sublethal effects on juvenile anadromous fish were uncertain. Currently, the application rate regulations for *Cascade*® are based on previously reported results found to be inconsistent and derived from inadequate sample sizes and studies employing fish in questionable conditions. For example, the State of Washington NPDES regulations are set at 2.5 ppm a.e., roughly two-thirds of the LOEC reported by Ligouri et al. (1983). These regulations are based on data from a seawater challenge study that employed small numbers of fish using a static system. Here we addressed a variety of factors in order to properly determine an accurate toxic response. We also paid meticulous attention to husbandry and water quality parameters. Advanced statistical techniques were used to ensure other variables were not affecting

comparisons of survival between experimental treatment groups. The following questions were addressed: (1) Does *Cascade*® exposure within the concentration range of 1-5 mg/L a.e. endothall reduce survival during seawater entry of salmon and steelhead smolts when accounting for potential effects of warm water temperatures (>18C) at the time of exposure; and, (2) is there interspecific variation in effects of endothall exposure on salmon and steelhead smolt survival during seawater entry?

Fish were exposed to various concentrations of *Cascade*® for four days in flow-through aquaria, allowed to recover for two days, and subjected to a ten-day seawater challenge. Results indicated that the threshold for exposure-related mortality during the seawater challenge was greater than 5 ppm a.e. endothall for all three species tested. Steelhead trout were generally most sensitive to saltwater entry and experienced the highest mortality during experimental trails. Statistical comparisons revealed that the difference between steelhead and salmon survival was significant for all treatments. Across all species, treatment groups exposed to 9-12 ppm a.e. endothall experienced significantly greater mortality than other treatment groups. Although increased survival was observed in some treatments >0 ppm a.e., compared with that observed in control, treatment groups between 0 and 8 ppm a.e. endothall did not differ statistically.

We found no observable effect of endothall during the 96-hour acute toxicity freshwater portion of the experiment at all concentrations tested (0-12 ppm a.e.). These results are consistent findings reported in the WDOE EIS for endothall, where they found the NOEC range at 41-51 ppm a.e. for rainbow trout.

However, the results yielded by our seawater challenge experiments revealed striking differences from some previously reported data. Liguori et al. (1983) indicated 100% mortality at Day-3 among juvenile Chinook within a 10-day seawater challenge following exposure to 3 ppm a.e. in freshwater. Toxic ammonia levels may be one influential factor that confounded the results they reported. According to the authors, total ammonia levels ranged between 0 and 1.9 mg/L, or approximately 0.07 mg/L unionized ammonia (NH_3). Unionized ammonia is potentially toxic at this level, and in preliminary trials we observed ammonia-induced mortality when NH_3 reached concentrations greater than or equal to this level (data not shown). Further, Liguori et al. (1983) employed static seawater challenge experiments, whereas a flow-through system was used for both phases of our experiment. In addition, only ten fish were used for each treatment group in the study reported by Liguori et al. (1983), without employing replicate experiments, which precluded statistical analysis. Researchers also reported using 4-gram Chinook salmon, substantially smaller than fish used in this study, which is another potential explanatory factor. Assuming Liguori et al. (1983) was reporting the average, or approximate size of fish used in their study, it is plausible that some of the specimens used during their trials were not physiologically prepared for seawater, which could explain the pattern in survival they reported – high mortality within 24-hours of seawater entry. We observed 100% survival during all trials for the first 72 hours in saltwater, even for fish treated >10 ppm a.e., while Liguori et al. (1983) reported 100% mortality within this same timeframe for fish treated at 3 ppm a.e.

Results from other studies such as Bouck and Johnson (1979) and Serdar and Johnson (1996) are perhaps less controversial because in the case of Bouck and Johnson (1979) two trials were completed each yielding a different result. Bouck and Johnson (1979) found that coho smolts exposed to 5 ppm a.e. endothall experienced 100% mortality after 10 days following direct transfer seawater, but they also observed 0% mortality in a replicate trial. Similarly, when coho were allowed to acclimate in freshwater prior to a gradual seawater challenge a range of 0 and 4% mortality was observed. The authors did not attempt to elucidate why they may have observed different results between trials. Similarly, Ligouri (1983) observed high mortality among juvenile Chinook salmon in a ten-day seawater challenge following a 48-hour exposure at 3 ppm a.e. in freshwater. Serdar and Johnson (1996) found no effect of 10 ppm a.e. endothall exposure on survival of coho salmon smolts subjected to a 24-hour seawater challenge. We suspect that inconsistent results between all three studies can be attributed to differences in experimental design.

A plasma sodium analysis serves as a sufficient index of osmoregulatory ability in seawater since sodium chloride is the major osmotic component (Clarke and Blackburn 1977). In our study, fish treated with *Cascade*® at various doses did not have elevated plasma sodium levels relative to control fish. Moreover, plasma sodium levels at Day-10 for coho and Chinook were similar to levels found in fish held in freshwater, indicating the ability of the fish to adapt to a saline environment and maintain proper regulation of their blood sodium concentrations. Our results are consistent with Clarke and Blackburn (1977)

where they found plasma sodium levels in coho salmon to significantly subside approximately 6-days following peak sodium levels observed 24-hours after saltwater entry. The increased plasma sodium concentration observed in steelhead (Table 4), compared with levels found in coho and Chinook, may be attributed to the difference in methodology for saltwater introduction for this particular trial (as reported in the *Methods*). Specifically, steelhead were brought to full-strength seawater by day-8 of the challenge, compared with the coho and fall Chinook by day-2. If the trial was allowed to progress to 14 days, we would expect plasma sodium concentrations to fall within the normal range for fish adapted to seawater. A change in the methodology for seawater introduction for the steelhead was employed because we observed a lower survival (but not significant) in the control fish (70%), compared with that observed with the Chinook (82%) and coho (82%). Further, it has been shown that steelhead are less efficient at maintaining ionic homeostasis at high salinities compared with Chinook (Morgan and Iwama, 1991). Therefore, in trial 4 we gradually increased salinity for the steelhead over a period of 8 days to account for their reduced ability to metabolically transition.

Cascade®, at chemical levels equivalent to the current Federally regulated level of 5 ppm a.e, does not appear to exhibit acute toxic effects (mortality) on coho, fall Chinook and steelhead in freshwater, and it does not affect their osmoregulatory capacity, as evidenced by results of our seawater challenge. Statistically significant differences observed between survival of test fish in the 0 and 8 ppm a.e. treatment groups and the 9-12 ppm a.e. treatment groups

suggest that the threshold for effects, or the lowest observed effect concentration (LOEC), of *Cascade*® on survival during seawater transition lies within the range of 9-12 ppm a.e., beyond the highest application concentration of 5 ppm a.e. Federally permitted.

In order to express an accurate dose-response relationship for *Cascade*®, we designed a sophisticated bioassay that considered physical, chemical and biological conditions that were overlooked by previous studies. In addition, a proper statistical analysis was employed to account for potential confounding factors and their effects on survival of different treatment groups. Further, we also provided evidence for necessary changes needed in standards for chemical testing regarding anadromous salmonids. Such critical factors that must be taken into consideration when conducting a seawater challenge experiment are methods of exposure (i.e. flow-through apparatus, length of toxicant and seawater exposure), specimen life-stage, water quality, and blood chemistry. The results of this multifaceted bioassay provide a credible basis for WDOE to determine a *Cascade*® application rate sufficient for the protection of anadromous salmonids. Based on our results, we anticipate no sublethal effects of Cascade® on salmon and steelhead when discharging at the federally labeled rate of 5 ppm a.e. endothall.

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APPENDIX A: SUPPLEMENTAL MATERIALS

Data Analysis

To facilitate statistical inference, statistical analysis relies on probability models that make assumptions about how data is collected. Therefore, the choice of one's statistical approach often has a major impact on the study design, and vise versa. The results of a statistical analysis may be unreliable if data collection is carried out in a way that biases observations, or an analytical approach is chosen that makes assumptions that are unsupported by data collection protocols. The primary issues that need to be addressed when working with data from toxicity testing are statistical independence among observations and whether groups of observations share a common variance. The EPA provides guidelines for addressing lack of common variance among groups of observations, but the analyses recommended by the EPA for non-statisticians (EPA 2002) assume the data is collected such that each observation is independent of all other observations in the dataset. This requirement, "statistical independence," is difficult to satisfy because data is often collected in a manner that implies there will be groups of observations with correlated outcomes, unrelated to experimental treatments. Even if a researcher does a thorough job of developing a randomized study design, there will likely be factors that inadvertently create groups of observations with correlated responses.

Our chosen analytical approach (GLMM) attempted to account for dependence between observations. This was essential because test animals were placed in seawater challenge tanks according to species to avoid competitive interactions between fish of different sizes and behavioral tendencies. GLMMs are commonly used in modern statistics and have been applied extensively in the field of toxicology. We recommend review of Noe et al. (2010) and Wheeler and Bailer (2009) if there is any question about the appropriateness of using a GLMM for analysis of data from toxicity studies. The GLMM also proved to be more useful for our study compared with some of the approaches proposed by EPA 2002 because the GLMM was sensitive to statistical differences between observations that a standard ANOVA was not able to detect. This is because covariance between observations from similar tanks and trials was accounted for by the GLMM.

The following paragraphs are intended to explain the similarities and differences between our statistical approach (GLMM) and analyses recommended by the EPA for non-statisticians[3]. We hope to demonstrate congruence between a

[3] Methods described by the EPA for analysis of toxicity test data are intended to provide examples of statistical tools approved for use and interpretation by non-statisticians. The EPA recommends working with a statistician when data does not meet assumptions of statistical tools outlined in EPA (2002). A statistician was responsible for conducting our data analysis, but this appendix was written with non-

GLMM and a standard ANOVA or T-test. During our exploration of these topics, we rely on Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (EPA 2002) for comparison between statistical approaches. For simplicity, we will refer to this as the EPA Methods document.

Addressing Statistical Assumptions

Section 11.1.6 of the EPA Methods document outlines how lack of independence in observations and outliers may affect the outcome of the statistical tools used to determine NOEC and LC50. In Section 11.1.6.1 it is stated, "*A critical assumption in the statistical analysis of toxicity data is statistical independence among observations. Statistical independence means that given knowledge of the true mean for a given concentration or control, knowledge of the error in any one actual observation would provide no information about the error in any other observation. One of the best ways to insure independence is to properly follow randomization procedures. The purpose of randomization is to avoid situations where test organisms are placed serially, by level of concentration, into test chambers, or where all replicates for a test concentration are located adjacent to one another, which could introduce bias into the test results.*" Due to potential competitive interactions between species, we did not randomize assignments of fish to tanks within specific trials and instead assigned fish of the same species to the same tanks. This violates the assumption of statistical independence between observations. The EPA Methods document provides no advice on how to alleviate the assumptions of statistical independence. We show later in this document how Generalized Linear Mixed Models (GLMM) can overcome this hurdle, but first start by showing the equivalence of a method recommended in the EPA Methods document, a T-test, to a simplified version of the model we used, linear regression.

Determining NOAEC with a T-test

Section of 11.3 of the EPA Methods document provides detailed information on four statistical approaches that can be used to determine the No Observed Adverse Effect Concentration (NOAEC). One approach may be favored over another depending upon whether the data is assumed to come from a normal distribution or if the variances of each of the treatment groups are equal. If the data is normal and the variances of each group are equal, one of the recommended approaches is a two sample T-test.
A two sample T-test tests the null hypothesis that the means of two normally distributed populations (or in this case samples from say a Treatment and Control group) are equal.

$$H_0 : \mu_{Treatment} = \mu_{Control}$$

---

statisticians in mind. As a result, some technicalities are over-simplified. To understand our approach, a statistician simply needs to know that we used a GLMM with nested random effects.

The details of the T-test are provided in section 11.3.5.4 of the EPA Methods document. It is important to note that the statistic provided in section 11.3.5.4.2 is T-distributed. If the p-value from the T-test (i.e. the area under the T-distribution curve) is small, then there is evidence to reject the null hypothesis.

Linear Regression

We will now show that a linear regression model with a categorical variable can be used, like a T-test, to test whether the means of two samples are equal. Linear models were not presented in the EPA Methods document and so here we provide some information, but do assume that the reader has some familiarity with the topic. In general, a linear regression model is expressed as

$$Y_i = \alpha + \beta \times X_i + \varepsilon_i$$

where α and β represent the intercept and slope respectively of the linear relationship between X_i and Y_i. i in this case, represents a single observation. ε_i is a random error term. A very critical assumption of this model is that ε_i is normally distributed with a mean of 0 and constant variance σ^2, $\varepsilon_i \sim$ Normal(0, σ^2). Furthermore, any two random error terms, ε_i and ε_j, are assumed to be uncorrelated so that their covariance is zero (as described in Section 11.1.6 of EPA Methods).

One common test in linear regression analysis is to assess whether there is a significant relationship between *X and Y*. This is achieved by testing the null hypothesis that

$$H_0 : \beta = 0$$

If we can reject this hypothesis then there is evidence to suggest a relationship between *X* and *Y*. In order to do this, we first need to find an estimate of β (i.e. $\hat{\beta}$). Once we have this estimate, we can test the above null hypothesis with the test-statistic

$$\frac{\hat{\beta} - \beta}{s(\hat{\beta})}$$

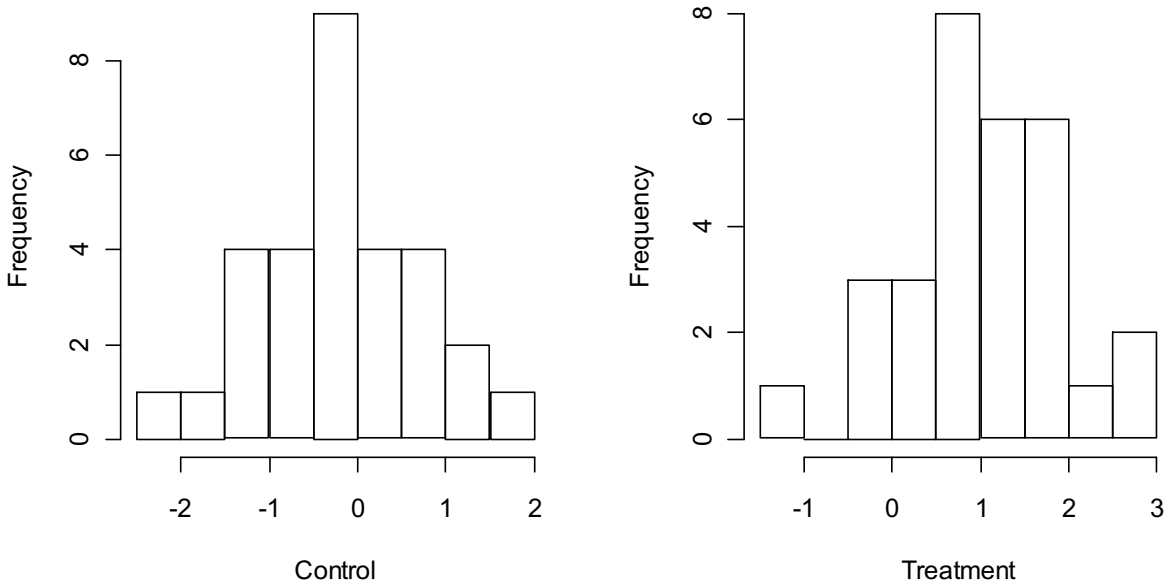
where $s(\hat{\beta})$ is the standard deviation of the estimate of β, $\hat{\beta}$. This test statistic is similar to the one provided in section 11.3.5.4.2 of the EPA Methods document in that it is also T-distributed. Likewise, if the p-value (i.e. the area under the T-distribution curve) is small, then there is evidence to reject the null hypothesis.

It's easiest to conceptualize a linear regression model between two continuous variables (e.g. height and weight), but the predictor variable, *X,* may also be categorical. If it is categorical, it is coded in the regression equation as an indicator variable. For example, if each observation *i* was either from a Treatment or Control group, then it would be coded as

$$X_i = \begin{cases} 1 & \text{if Treatment} \\ 0 & \text{if Control} \end{cases}$$

When *X* is categorical, it's associated slope coefficient, β, shows how much higher (or lower) the mean response line is for the sample coded 1 than the line for sample coded 0. This interpretation is the important link between linear regression with categorical variables and two sample T-tests.

We will now provide an example of the above concepts. Let's say and experiment was conducted with a Control and Treatment group. Histograms of the variable measured in the experiment for the Treatment and Control group are provided below.



The mean of Treatment and Control groups were computed with the R Statistical Software

```
> mean(Treatment)
[1] 1.046972
> mean(Control)
[1] -0.2105601
```

Based on visual inspection, we would assume that each of the samples are normal and have about the same variance. Since these assumptions are met, we could use a two sample T-test. Let's first start with a linear regression model. A sample of the data formatted for the regression model is shown below:

```
Y           X
-0.1100702  1
-1.4706440  1
0.8181937   1
. . . . .
0.54660220  0
0.69629735  0
1.57322191  0
```

We'll fit the linear regression model using the R Statistical Software.

```
> linearModel = lm(Y ~ X)
> summary(linearModel)$coefficients
              Estimate Std. Error   t value     Pr(>|t|)
(Intercept) -0.2105601  0.1630026 -1.291759 2.015646e-01
X            1.2575320  0.2305205  5.455185 1.056807e-06
```

And we'll conduct a two-sample T-test using the R Statistical Software

```
> t.test(Treatment, Control, var.equal=TRUE)
t = -5.4552, df = 58, p-value = 1.056807e-06
alternative hypothesis: true difference in means is not equal to 0
```

The p-values from the two models are highlighted in yellow. Note that they are identical. The estimated β is highlighted in green. Note that the difference between the mean Treatment and mean Control (1.046972 - -0.2105601)is identical to the estimate of β. A linear regression model with categorical variables is equivalent to a two-sample T-test and is a viable way to conduct acute toxicity analysis.

Linear Regression with Mixed Effects

We can rewrite the above linear regression model as values in a matrix

$$\begin{bmatrix} Y_1 \\ Y_2 \\ \cdot \\ \cdot \\ Y_n \end{bmatrix} = \begin{bmatrix} 1 & X_1 \\ 1 & X_2 \\ & \cdot \\ & \cdot \\ 1 & X_n \end{bmatrix} \begin{bmatrix} \alpha \\ \beta \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \cdot \\ \cdot \\ \varepsilon_n \end{bmatrix}$$

A compact way to write this in matrix terms is as follows

$$\mathbf{Y = X\,\beta + \varepsilon}$$

The **boldface** symbol indicates a matrix.

A critical assumption of the linear regression model presented above was that ε_i is normally distributed with a mean of 0 and constant variance σ^2, $\varepsilon_i \sim$ Normal(0, σ^2) and that any two random error terms, ε_i and ε_j, are assumed to be uncorrelated so that their covariance is zero. This can be expressed in matrix terms through the variance-covariance matrix

$$\boldsymbol{\sigma^2\{\varepsilon\}} = \begin{bmatrix} \sigma^2 & 0 & 0 & \cdots & 0 \\ 0 & \sigma^2 & & \cdots & \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & 0 & \cdots & \sigma^2 \end{bmatrix}$$

The constant variance assumption is expressed in the diagonal elements of the matrix (i.e. all the diagonal elements have the same value, σ^2, and the uncorrelated assumption is expressed in the off-diagonal elements which express the covariance between any two observations (i.e. since all the covariances between any two observations are zero, their correlation is zero).
In toxicological studies, there are many reasons why observations may not share a common variance and may be correlated. If groups of observations do not share a common variance, a test is provided in the EPA Methods Section 11.3.5.6. However, a shortcoming of this method is that it still assumes all errors are uncorrelated, which we know is no true in our study. Linear Mixed effects Models (LMMs) will allow us to overcome this hurdle, while still providing valid statistical comparisons between groups (i.e. Treatment and Control).
In short, LMMs achieve this is by modifying the above variance-covariance matrix. For simplicity, let's assume that that all observations had the same common variance, but that the covariance between any two observations is d^2.
This is expressed in variance-covariance matrix by:

$$\boldsymbol{\sigma^2\{\varepsilon\}} = \begin{bmatrix} \sigma^2 + d^2 & d^2 & d^2 & \cdots & d^2 \\ d^2 & \sigma^2 + d^2 & d^2 & \cdots & d^2 \\ \vdots & \vdots & \vdots & \ddots & \vdots \\ d^2 & d^2 & d^2 & \cdots & \sigma^2 \end{bmatrix}$$

Specifically, LMMs add an additional random effect term that induces additional variance and correlation into the variance-covariance matrix.

$$\mathbf{Y = X\,\beta + Zb + \varepsilon}$$

In the above example

$$\boldsymbol{Zb} = \begin{bmatrix} \mathbf{1} \\ \mathbf{1} \\ . \\ . \\ \mathbf{1} \end{bmatrix} [\boldsymbol{d^2}]$$

This is the general idea behind LMMs: instead of transforming the data to deal with heterogeneity or questioning the data because of violations of independence, LLMs account for heterogeneity or lack of independence by modeling the distribution of the error terms in the linear model.

LMMs are a challenging topic, but are a powerful tool when assumptions of simpler statistical tools are violated. The above explanation is very cursory and there is a wealth of information on this topic available on the web. An excellent introductory book is, Mixed Effects Models and Extension in Ecology with R (Alain F. Zurr and others).

General Linear Regression with Mixed Effects

The General Linear Regression Model (GLM) (e.g. logistic regression) is an extension of the linear regression model (without random effects) discussed above. Recall the linear regression model:

$Y_i = \alpha + \beta \times X_i + \varepsilon_i$

If we wanted to predict survival, Y_i, from some predictor X_i, it's tempting to use the above model. However, since the response variable is survival we need some way to constrain the predictions between zero and one. For instance, if an estimated model of this form predicted that survival is 0.0 at a lethal dosage level, the model would then predict that survival is < 0.0 at dosage level greater than the lethal dosage level because the relationship is linear. This is clearly non-sense because survival cannot be less than 0. Note, however, that if $0 < Y_i < 1$, then $\frac{Y_i}{1-Y_i} > 0$ and $-\infty < \log\left(\frac{Y_i}{1-Y_i}\right) < \infty$. The ratio $\frac{Y_i}{1-Y_i}$ is referred to as the odds ratio. If we transform survival by the log-odds ratio in the regression equation constraining its values between 0 and 1, then any value predicted by $\alpha + \beta \times X_i + \varepsilon_i$ would yield a legitimate prediction.

$$\log\left(\frac{Y_i}{1-Y_i}\right) = \alpha + \beta \times X_i + \varepsilon_i$$

Like the linear regression model, if X is categorical, it's associated slope coefficient, β, shows how much higher (or lower) the mean response line is for the sample coded 1 than the line for sample coded 0. The mean response line is now in log-odds space so we must back transform it to obtain more informative predictions. In particular

$$Y_i = \frac{e^{\alpha + \beta \times X_i}}{1 + e^{\alpha + \beta \times X_i}}$$

The concept of relating linear predictors to some function of a mean response forms the basis of general linear models. This includes Logistic, Poisson, Multinomial regression to name a few. In the context of our analysis, we used logistic regression to relate species and endothall exposure to survival.
What about the extra M in GLMM ? As you might expect, a GLMM is a general linear model with mixed effects terms. When random effects are modeled, the logit function above is rewritten as

$$\log\left(\frac{Y_i}{1-Y_i}\right) = \alpha + \beta \times X_i + b \times Z_i + \varepsilon_i$$

where $b \times Z_i$ could be some random effect component. The principles described for the LMM apply to the GLMM – by adding a random effect component were inducing more variation and correlation into the variance-covariance matrix of the error component to either adequately model heterogeneities in group variances or dependence between observations in the study, or both.

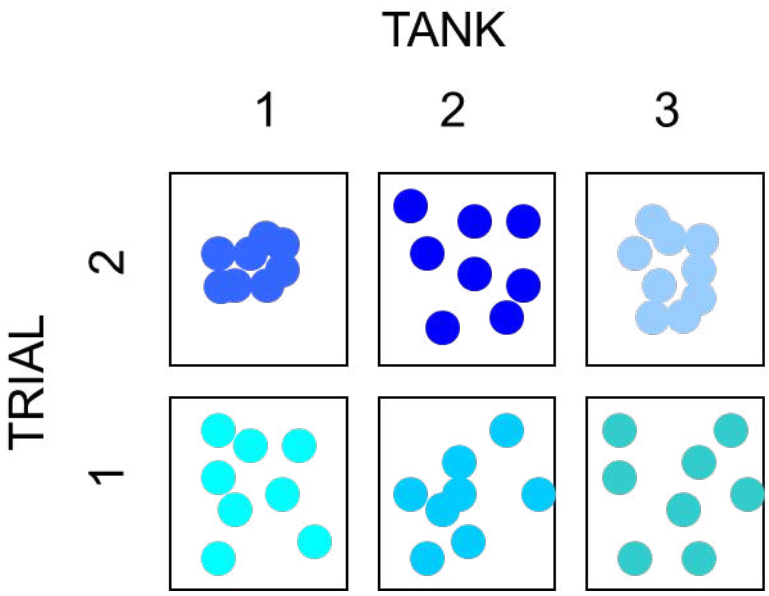
The Nested Random Effect Design

In our study we chose to model nested random effects between tanks and trial. In our report this was represented as

$a_i \times b_j$

when describing the candidate set of models that we would select amongst using AIC. No design matrix (i.e. Z_i) was presented because technically these random effects were modeled as intercepts and inclusion of that matrix is not necessary (that is, the matrix consists entirely of 1's). A nested random effect structure induces a complicated structure into the variance-covariance matrix. Here we try to present this structure with a simple example.

Suppose a study consisted of two trials and the same three tanks were used in each trial as represented below.



The proximity of the dots (observations) within a tank and trial is meant to represent the variability within each group. The color of each observation represents an unexplained similarity. Due to some unexplained reason, two observations of the same color are more likely to be alike compared to two observations of different colors. In the above representation, clearly tank 1 within trial 2 had much less variability than tank 1 in trial 1, indicated by the close proximity of dots in column 1, row 1. Assuming a common variance would likely underrepresent the true variability between these observations. Let's assume there were uncontrolled factors in the study that varied between each tank within a trial. For example, although tank 1 was used in both trial 1 and trial 2, we could hypothesize that the ability to precisely control some variable, such as water flow, might have varied slightly in the two trials. If this were the case, the outcome of observations in trial 2, tank 1 are more likely to be similar to each other compared to observations in trial 1, tank 1 – even though the same tank was

used. The two salient features of the data represented above are: relatedness of observations within groups and a lack of common variance between groups. There are several ways to address the issue of a lack of common variance in T-tests and ANOVA, but there is no way to model relatedness between observations. A GLMM with a nested random effect structure accounts for the two features of the data represented above, which are relatedness of observations within a trial and tank and variability of those observations for each unique combination of trial and tank. That is, the assumptions of the model are consistent with the features of the data.

Additional Statistics

This section is intended to provide additional statistical support to aid regulatory decision-making. WDOE expressed concern about the General Liner Mixed Effects Models (GLMMs) used in the main body of the report to determine the No-observed-adverse-effect concentration (NOAEC). Section 11 of Acute Toxicity Data Analysis of Methods of Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (EPA 2002) provides basic statistical guidelines for determining NOAEC. Although the GLMM approach is the most rigorous treatment of the data, it is not mentioned in the EPA guidelines. Here we provide additional analysis based on the guidelines of Section 11, EPA 2002. The p-values from the two-sample comparison tests are presented below by species. The two-sample comparison method depended on assumptions about the data (normality and homogeneity of variance) as determined by the decision tree on page 87 of EPA 2002. For reproducibility, R statistical software code that yields these results can be provided upon request.

Chinook

There is no evidence to suggest that the mean survival for chinook at any endothall level is statistically different than mean survival for fish in the control group (Family-wise error rate, $\alpha = 0.05 / 5 = 0.01$). The test statistic and p-values for all pairwise comparisons from the T-tests are shown below. All comparisons are made against the Control, 0 ppm endothall.

Group	t (test statistic)	p-value
4.83 ppm	0.648	0.5282
5.43 ppm	1.5635	0.1439
7.58 ppm	0.1832	0.8574
9.24 ppm	0.7142	0.4914
11.61 ppm	2.851	0.0172

Coho

There is no evidence to suggest that the mean survival for coho at any endothall level is statistically different than mean survival for fish in the control group (Family-wise error rate, $\alpha = 0.05 / 5 = 0.01$). The test statistic and p-values for all pairwise comparisons from the Wilcoxon Rank Sum tests are shown below. All comparisons are made against the Control, 0 ppm endothall.

Group	W (test statistic)	p-value
4.83 ppm	15	1.0000
5.43 ppm	19	0.9421
7.58 ppm	18.5	0.8850
9.24 ppm	22	0.2652
11.61 ppm	27	0.0490

Steelhead

There is no evidence to suggest that the mean survival for steelhead at any endothall level is statistically different than mean survival for fish in the control group (Family-wise error rate, $\alpha = 0.05 / 3 = 0.0167$). The test statistic and p-values for all pairwise comparisons from the Wilcoxon Rank Sum tests are shown below. All comparisons are made against the Control, 0 ppm endothall.

Group	W (test statistic)	p-value
3.77 ppm	7	0.6714
6.17 ppm	6	0.4795
9.24 ppm	17.5	0.0349

Raw Data

Table 1. Raw data of endothall concentrations from freshwater samples taken at 24 and 72 hours following toxicant administration, as measured by U.S. EPA method 548.1 (Anatek Labs, Moscow ID).

TANK 1	Species	Objective (ppm a.e)	24 h (ppm a.e)	72 h (ppm a.e)	Mean (ppm a.e)
Trial 1	SH, CO, FC	0	ND	ND	0
Trial 2	CO, FC	0	ND	0.07	0.04#
Trial 3	CO, FC	0	ND	ND	0
Trial 4*	SH	0	ND	0.02	0.01#

TANK 2	Species	Objective	24 h	72 h	Mean
Trial 1	SH, CO, FC	10	9.42	9.06	9.24
Trial 2	CO, FC	5	4.69	4.97	4.83
Trial 3	CO, FC	5	5.68	5.18	5.43
Trial 4*	SH	3.5	3.56	3.98	3.77

TANK 3	Species	Objective	24 h	72 h	Mean
Trial 1	N/A	N/A	N/A	N/A	N/A
Trial 2	CO, FC	10	9.71	13.50	11.61
Trial 3	CO, FC	7.5	7.55	7.60	7.58
Trial 4*	SH	5	6.58	5.76	6.17

ND = not detected; SH = steelhead; CO = coho; FC = fall Chinook; N/A = not applicable, no fish were used. #Denotes values that were assumed as 0 (zero) in statistical analysis. *For trial 4, steelhead were subjected to a slower transition time into full strength seawater. Steelhead experience full strength seawater for days 8-10, see Methods.

Table 2. Raw data of percent daily survival (%) of coho, chinook, and steelhead following exposure to Cascade and subjection to a 10-day seawater challenge.

	Trial	dose (ppm a.e.)	n	1	2	3	4	5	6	7	8	9	10
SH	1	0	30	100	100	100	97	93	87	77	73	70	70
	1	9.24	30	100	100	100	97	63	57	53	43	37	37
	4*	0	27	100	100	100	100	100	100	100	100	100	100
	4*	3.77	27	100	100	100	100	96	96	93	93	93	93
	4*	6.17	27	100	100	100	96	96	96	96	96	96	96
CO	1	0	30	100	100	100	93	87	87	87	83	80	80
	1	9.24	30	100	100	100	97	87	80	73	70	70	70
	2	0	29	100	100	100	97	86	76	76	76	76	76
	2	4.83	30	100	100	100	97	87	87	87	87	87	87
	2	11.61	26	100	100	96	88	62	50	50	50	50	50
	3	0	20	100	100	100	100	100	95	95	95	95	90
	3	5.43	21	100	100	100	100	86	86	86	86	86	86
	3	7.58	19	100	100	100	100	95	95	95	95	95	89
FC	1	0	30	100	97	93	93	90	87	87	87	87	87
	1	9.24	30	100	97	93	83	83	80	77	77	77	77
	2	0	29	100	100	100	97	86	76	76	76	76	76
	2	4.83	31	100	100	97	97	97	84	84	84	84	84
	2	11.61	31	100	100	94	87	71	61	61	61	61	61
	3	0	30	97	97	97	97	93	90	83	83	83	83
	3	5.43	24	100	100	100	100	100	100	79	79	70	70
	3	7.58	27	100	100	100	100	100	100	89	89	89	85

SH = steelhead; CO = coho; FC = fall Chinook. n = the total number of fish at the beginning of the acute toxicity experiment. *For trial 4, steelhead were subjected to a slower transition time into full strength seawater. Steelhead experience full strength seawater for days 8-10, see Methods.

Table 3. Raw data of plasma sodium concentrations, as measured by indirect potentiometry (Oregon Health & Science University, Portland OR).

	Trial	Dose (ppm a.e.)	Replicate 1 (mmol/L)	Replicate 2 (mmol/L)	Replicate 3 (mmol/L)	Total mean ± SD (mmol/L)
SH	4*	0	207	186	194	196 ± 10.83
	4*	3.77	194	180	192	188 ± 7.29
	4*	6.17	165	187	186	179 ± 12.58
CO	3	0	150	157	160	156 ± 5.13
	3	5.43	154	155	162	157 ± 4.25
	3	7.58	161	160	163	161 ± 1.26
FC	3	0	147	152	156	152 ± 4.77
	3	5.43	164	159	N/A	162 ± 3.54
	3	7.58	154	152	153	153 ± 5.39
Freshwater	--	--	140	156	N/A	148 ± 11.31

SH = steelhead; CO = coho; FC = fall Chinook; N/A = not applicable; blood sample triplicate was not collected. Total mean values represent the mean of the three combined replicates. *For trial 4, Steelhead were subjected to a slower transition time into full strength seawater. Steelhead experience full strength seawater for days 8-10, see Methods. Plasma sodium levels reported represent data obtained from FC only.

Table 4. Weight and length data over all trials for all species.

Species	n (Beginning of toxicity, seawater experiments)	Mean Weight (g) [range]	Mean Length (mm) [range]
Steelhead	(142,142)	152 ± 12.9 [130-181]	246.5 ± 5.8 [238-256]
Coho	(209,203)	59.64 ± 10.2 [44-78]	176.27 ± 10.1 [157-191]
Chinook	(242,242)	13.3 ± 3.3 [8-17]	110.9 ± 11.4 [93-125]

APPENDIX B: R GLMM OUTPUT

R statistical output provided by the lme4 package for Model 2.

```
> model2
Generalized linear mixed model fit by the Laplace approximation
Formula: alive ~ species + dose.lump + (1 | trial/tank)
   Data: dat.glm
   AIC   BIC logLik deviance
 575.5 610.4 -279.8    559.5
Random effects:
 Groups     Name        Variance   Std.Dev.
 tank:trial (Intercept) 2.2619e-10 0.00001504
 trial      (Intercept) 9.9811e-01 0.99905627
Number of obs: 577, groups: tank:trial, 28; trial, 4

Fixed effects:
                 Estimate Std. Error z value Pr(>|z|)
(Intercept)       2.18921    0.56528   3.873 0.000108 ***
speciescoho      -0.03715    0.23834  -0.156 0.876123
speciessteelhead -1.06268    0.35995  -2.952 0.003154 **
dose.lumpLow      0.02176    0.31895   0.068 0.945615
dose.lumpMedium   0.34778    0.49199   0.707 0.479639
dose.lumpHigh    -1.07964    0.26186  -4.123 3.74e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) spcsch spcsst ds.lmL ds.lmM
speciescoho -0.203
specisstlhd -0.266  0.333
dose.lumpLw -0.213 -0.018  0.018
dose.lmpMdm -0.170  0.004  0.001  0.303
dose.lmpHgh -0.201  0.014  0.088  0.318  0.109
```