Final Report on Pacific Herring (*Clupea pallasi*) Test Development and Validation

with an Appendix on Herring Embryo Temperature Tolerance Comparisons between West Coast Stocks

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Cover photos by Paul Dinnel at the Shannon Point Marine Center
Left: Normal larvae at hatch
Right: Abnormal larvae at hatch

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Final Report on Pacific Herring (*Clupea pallasi*) Test Development and Validation

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by

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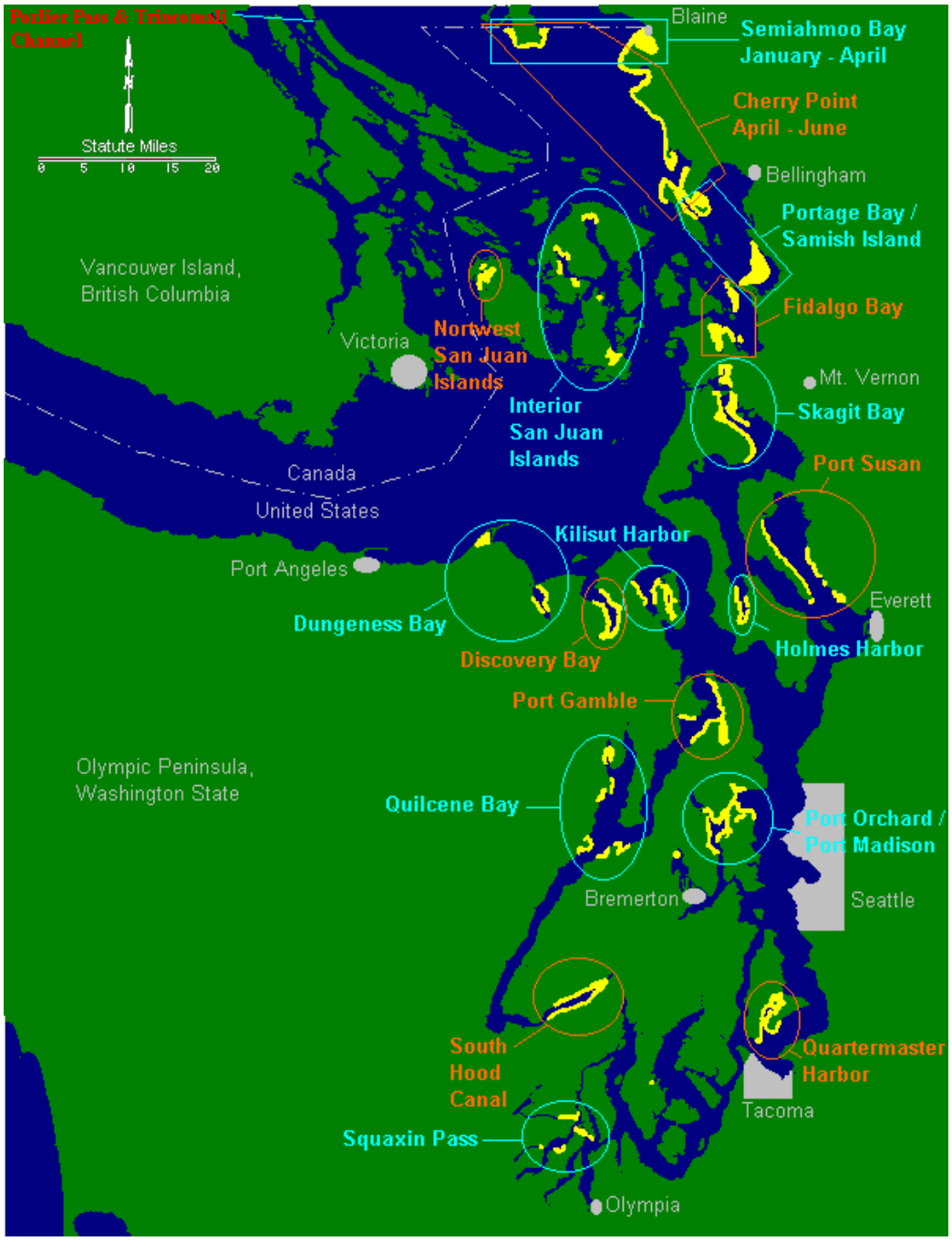

Documented Herring Spawning Grounds in Washington State
Washington Department of Fish and Wildlife

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Introduction

Because of the serious and ongoing decline in the population of the state's once largest herring stock, the Department of Ecology and Western Washington University have been developing and validating herring toxicity tests since the 2000 spawning season. The effort has cost close to $870,000 so far. The Department of Ecology provided 45% of these funds, industry provided 40%, and the remaining 15% of the funding came from grants. The herring test development effort has produced methods for a 96-hour acute survival test, an embryo survival & development test, and a larval 7-day survival & growth test. A description of the method development and final detailed protocols was recently published in the peer-reviewed journal, **Archives of Environmental Contamination and Toxicology**. (Dinnel et al, 2011)

Paul Dinnel is the lead researcher for the project and works at the Shannon Point Marine Center in Anacortes, WA. Nautilus Environmental in Fife, WA also contributed to method development and successfully completed the validation exercise for all three herring tests and all three reference toxicants. Nautilus Environmental in Burnaby, BC has successfully completed herring chronic method validations using copper chloride. The King County Environmental Lab participated in some of the method development trials, but never completed the validation exercise. New Fields in Port Gamble, WA recently reported copper chloride results for the herring 7-day survival and growth test. This report has been updated from the original November 2011 publication in order to incorporate the New Fields results.

The eight documents listed in the references with Dr. Paul Dinnel as the primary author describe the process of herring test development. Many combinations of test duration, test chamber type and size, feeding routines, salinities, test temperatures, etc. were tried over the years in order to find the optimal test conditions for the three herring tests. The final herring test protocols are described in Dinnel et al, 2011. Lists of the herring test conditions are also included in the Department of Ecology's ***Laboratory Guidance and Whole Effluent Toxicity Test Review Criteria***. (Marshall, 2008)

Background

The Cherry Point herring stock has been a great concern to Washington State in recent years. It once had a spawning biomass equal to that of all of the other herring stocks in the state combined. According to Washington Department of Fish and Wildlife (WDFW) annual spawning surveys (Stick et al, 2009), the Cherry Point stock size declined from nearly 15,000 tons of spawning biomass in 1973 to just above 800 tons in 2000. Stock size rose gradually until 2007 when it began declining again and dropped below 800 tons in 2010.

Recruitment is the number of first time spawners and is a direct measure of the success of reproduction two years earlier. Recruitment from 1974 to 1995 averaged 2121 tons. 1994 had a record recruitment of 4076 tons. However, recruitment dropped steeply in 1996 and only averaged 755 tons from 1996 to 2001. Recruitment in 2004 was only 22 tons. Herring deposit eggs nearshore where human activities can be a significant factor in environmental quality.

Even though the available evidence points to other causes such as malnutrition and disease as being key factors in the Cherry Point herring decline, permit writers included herring toxicity testing requirements in industry National Pollutant Discharge Elimination System (NPDES) permits in the Cherry Point area in order to rule out effluent toxicity as the cause of the decline in recruitment. Requirements to test with herring have also been put into municipal wastewater plant permits near Cherry Point and into permits for industry discharges in other locations with vulnerable herring stocks.

Topsmelt (*Atherinops affinis*) is EPA's standard fish species for larval survival & growth testing on the West Coast. A limited amount of testing with anonymous oil refinery and aluminum smelter effluent samples showed the 7-day larval herring survival & growth test to be more sensitive than topsmelt in 3 out of 4 tests. (Dinnel et al, 2008)

The herring embryo test was more sensitive than the echinoderm (sea urchin, *Strongylocentrotus purpuratus*, or sand dollar, *Dendraster excentricus*) embryo test in 1 out of 4 industry effluent tests, but the echinoderm test results were more sensitive in 2 out of 4 tests. (Dinnel et al, 2008) Earlier comparison testing established that herring embryos were more sensitive to creosote leachate than echinoderm embryos. (Dinnel et al, 2006)

Industries have been routinely monitoring effluent discharges since 2007 using the 96-hour herring acute survival test. Some of these samples were also tested with 96-hour fathead minnow or topsmelt acute tests. See Table 2 for all of the acute test results. The 96-hour acute survival test with herring was more sensitive than topsmelt in 2 out of 13 tests on industry effluent samples in the agency CETIS database, but the topsmelt acute test was more sensitive twice as often. The fathead minnow 96-hour acute survival test was more sensitive than herring in 2 out of seven tests on industry effluent while herring were more sensitive in none of those 7 tests.

Dr. Dinnel is currently working on a manuscript for a more detailed article on herring versus EPA test comparisons to be published in a peer-reviewed journal.

Analysis of Validation Data

We agreed with industry at the beginning of this project that a herring test would be considered to be validated for regulatory use when a commercial lab demonstrated the ability using three reference toxicants to get no more than a 60% coefficient of variation (CV) in point estimates at the 25% effect level and sufficient power to differentiate a minimum significant difference of less than or equal to 40 percent between control and treatment groups at a statistical power of 80 percent (alpha = 5 percent, beta = 20 percent). The most convenient measure of statistical power for this purpose is the percent minimum significant difference (PMSD) calculated by CETIS (Tidepool Scientific, 2010) for every control comparison. We also specified that a meaningful concentration-response relationship was a validation criterion and that point estimates at the 50% effect level would be considered, especially when data would not support calculation of the 25%

effect level. The three reference toxicants chosen for the validation exercise were copper chloride ($CuCl_2$), potassium chloride (KCl), and sodium dodecyl sulfate (SDS).

The control comparisons and point estimates in this report were produced according to recommendations in EPA toxicity test manuals and analyzed using CETIS v1.8.0.4. The conditions for running linear regression to derive point estimates were not met by several test datasets and missing point estimates would be a problem because of the relatively low number of test results available for analysis. In addition, linear regression and its alternative in the EPA manuals, Spearman-Kärber, can produce point estimates that are quite different from the same dataset and should not be combined into the same assessment of variability. For these reasons, Spearman-Kärber was used to calculate all median effect (LC50 or EC50) results for quantal data sets. Linear regression produced all 25% effect (LC25 and EC25) point estimates for quantal datasets. Linear interpolation provided both 25% and 50% (IC25 and IC50) point estimates for nonquantal data.

Results

Ninety-six-hour acute survival tests

The 96-hour acute survival test results from Nautilus Environmental in Fife are shown in Table 1 (reference toxicants) and Table 2 (industry effluents). These results show:

- The coefficients of variation for the LC25s and LC50s from the three reference toxicants were all below 60%. The highest CV was 41% for the copper chloride LC25s.
- The average PMSD for the reference toxicant data was 17.8%. Only 1 of the 16 reference toxicant acute test PMSDs (52.9%) was over 40%.
- In the acute results from testing with industry effluents, the mean PMSD for fathead minnows was 10.7%, for herring was 12.8%, and for topsmelt was 20.4%. One herring PMSD (40.1%) was over 40%, but topsmelt also had a PMSD of 40.0% in one of the thirteen tests conducted with this standard EPA species.

Table 1. 96-hour Acute test reference toxicant results

Test Code	Start Date	Reference Toxicant	Control Comparisons			Point Estimates	
			NOEC	LOEC	PMSD	LC25	LC50
NEFacute01	3/18/2005	Copper chloride	600	> 600	4.3%	NC	NC
NEFacute02	3/22/2005	Copper chloride	1000	2000	52.9%	1086.6	1447.3
NEFacute03	3/28/2005	Copper chloride	1000	2000	19.2%	1337.2	1893.5
NEFacute04	4/5/2005	Copper chloride	500	1000	23.3%	533.6	749.2
NEFacute05	5/18/2005	Copper chloride	1000	2000	13.6%	1335.6	1802.5
NEFacute06	5/19/2005	Copper chloride	1000	2000	9.9%	1952.7	2256.0
			Coefficient of Variation			**0.41**	**0.35**

Test Code	Start Date	Reference Toxicant	Control Comparisons			Point Estimates	
			NOEC	LOEC	PMSD	LC25	LC50
NEFacute07	3/18/2005	Potassium chloride	250	500	9.1%	525.9	637.3
NEFacute08	4/5/2005	Potassium chloride	250	500	8.9%	600.2	677.6
NEFacute09	5/18/2005	Potassium chloride	500	1000	4.5%	NC	707.1
NEFacute10	5/19/2005	Potassium chloride	500	1000	7.5%	NC	671.3
NEFacute11	6/3/2005	Potassium chloride	400	800	23.3%	796.0	857.4
			Coefficient of Variation			**0.22**	**0.12**

Test Code	Start Date	Reference Toxicant	Control Comparisons			Point Estimates	
			NOEC	LOEC	PMSD	LC25	LC50
NEFacute12	3/20/2005	Sodium dodecyl sulfate	5	10	20.5%	NC	9.1
NEFacute13	4/5/2005	Sodium dodecyl sulfate	5	10	37.0%	NC	7.1
NEFacute14	5/18/2005	Sodium dodecyl sulfate	2.5	5	31.5%	NC	4.4
NEFacute15	5/18/2005	Sodium dodecyl sulfate	2.5	5	8.9%	4.9	5.5
NEFacute16	6/3/2005	Sodium dodecyl sulfate	5	10	10.7%	5.6	6.9
			Coefficient of Variation			**0.09**	**0.27**

Table 2. 96-hour acute test effluent monitoring history

Industry Facility	Sample Collection	Test Organism	Biological Endpoint	Control Comparisons NOEC	LOEC	PMSD	LC50
Aluminum Smelter	2/26/2007	Pacific herring	96-hour survival	100	> 100	2.5%	
Oil Refinery 1	1/17/2007	Pacific herring	96-hour survival	100	> 100	12.0%	
	2/19/2007	Pacific herring	96-hour survival	100	> 100	2.5%	
	3/4/2009	Pacific herring	96-hour survival	100	> 100	9.9%	
	3/26/2009	Pacific herring	96-hour survival	25	50	8.5%	74.9
	4/6/2010	Pacific herring	96-hour survival	100	> 100	25.1%	
	1/31/2011	Pacific herring	96-hour survival	25	50	12.6%	62.9
	2/24/2011	Pacific herring	96-hour survival	100	> 100	11.0%	
Oil Refinery 2	2/20/2007	Pacific herring	96-hour survival	100	> 100	7.6%	
		topsmelt	96-hour survival	100	> 100	11.3%	
	3/28/2007	Pacific herring	96-hour survival	100	> 100	13.8%	
		topsmelt	96-hour survival	100	> 100	19.9%	
	3/6/2009	Pacific herring	96-hour survival	100	> 100	7.4%	
	3/30/2009	Pacific herring	96-hour survival	100	> 100	15.6%	
	4/7/2010	Pacific herring	96-hour survival	100	> 100	18.9%	
	2/24/2011	Pacific herring	96-hour survival	100	> 100	8.0%	
Oil Refinery 3	1/30/2007	fathead minnow	96-hour survival	50	100	12.3%	76.9
		Pacific herring	96-hour survival	100	> 100	6.7%	
		topsmelt	96-hour survival	3.6	100	17.8%	> 100
	6/11/2007	fathead minnow	96-hour survival	50	100	20.2%	66.9
		Pacific herring	96-hour survival	50	100	13.3%	69.7
		topsmelt	96-hour survival	50	100	22.7%	70.7
	5/21/2008	fathead minnow	96-hour survival	100	> 100	10.1%	
		Pacific herring	96-hour survival	100	> 100	40.1%	
		topsmelt	96-hour survival	100	> 100	22.6%	
	2/27/2009	fathead minnow	96-hour survival	100	> 100	5.9%	
		Pacific herring	96-hour survival	100	> 100	6.7%	
		topsmelt	96-hour survival	100	> 100	5.0%	
	3/26/2009	Pacific herring	96-hour survival	50	100	11.6%	81.3
	4/6/2010	fathead minnow	96-hour survival	50	100	15.0%	> 100
		Pacific herring	96-hour survival	50	100	18.8%	> 100
		topsmelt	96-hour survival	12.5	25	27.0%	45.6
	2/1/2011	fathead minnow	96-hour survival	100	> 100	7.1%	
		Pacific herring	96-hour survival	100	> 100	12.5%	
		topsmelt	96-hour survival	100	> 100	25.3%	
	2/24/2011	fathead minnow	96-hour survival	50	100	6.2%	> 100
		Pacific herring	96-hour survival	50	100	17.3%	99.6
		topsmelt	96-hour survival	50	100	26.1%	63.0
Oil Refinery 4	1/30/2007	Pacific herring	96-hour survival	100	> 100	6.5%	
		topsmelt	96-hour survival	100	> 100	14.5%	
	5/21/2008	Pacific herring	96-hour survival	25	100	21.0%	48.7
		topsmelt	96-hour survival	50	100	40.0%	64.5
	2/27/2009	Pacific herring	96-hour survival	100	> 100	10.6%	
		topsmelt	96-hour survival	100	> 100	11.3%	
	4/7/2010	Pacific herring	96-hour survival	100	> 100	14.2%	
		topsmelt	96-hour survival	50	100	22.2%	> 100
	2/2/2011	Pacific herring	96-hour survival	100	> 100	12.1%	

Larval 7-day survival and growth test

The herring 7-day survival & growth test results from the Nautilus Environmental lab in Fife using all three reference toxicants are shown in Table 3. These results show:

- All of the CVs for the Nautilus, Fife 7-day survival & growth tests were under 60%. The 25% effect and 50% effect point estimate CVs for all three reference toxicants and all three endpoints (survival, biomass, and weight) were under 60%.

- Replicate 4 of 100 ug/L was excluded from the 4/5/2010 copper chloride test because of being an outlier. If the result from this test chamber had been included, the CV for the biomass IC25 would have been 70%. The CV for the biomass IC50 was 15%, even with the outlier included.
- The outlier was included in the weight calculations. With replicate 4 of 100 ug/L included, the standard deviation for 7-day survival at this concentration was 0.22 and Ecology Publication WQ-R-95-80 requires a switch to the weight endpoint when survival standard deviations exceed 0.20.
- The LOEC remained the same whether replicate 4 of 100 was included or not. Because control comparisons are used for effluent monitoring, the LOEC is a more pertinent consideration.
- All of the PMSDs for each reference toxicant and endpoint were less than 40%.

The herring 7-day survival & growth test results with copper chloride from the Shannon Point Marine Center, Nautilus, Burnaby, and New Fields are shown in Table 4. These results show:

- The Shannon Point Marine Center produced a CV for the LC25 that was 68% and a CV for the biomass IC25 that was 92%. The CV for the weight IC25 was 48%. The CV for the LC50 was 48% and the CV for the biomass IC50 was 39%.
- The Nautilus, Burnaby lab produced CVs that were all under 30% for the 25% effect and 50% effect point estimates from all three endpoints in the 7-day survival & growth tests.
- New Fields produced CVs under 60% for 7-day survival and for weight. The CV for the biomass IC25 was 77%, but the CV for the biomass IC50 was 37%. Three of the four biomass NOEC - LOEC pairs were identical including those from the test with the biomass IC25 causing the CV exceedance.
- All of the PMSDs from the Shannon Point Marine Center, Nautilus, Burnaby, and New Fields 7-day survival & growth tests were under 40%.

Figure 1 - Herring Larvae following a 12-day Exposure to Copper

The copper exposure concentrations ranged from 0 µg/liter for the herring at the top of the photo to 750 µg/liter for those at the bottom. Note the progressively smaller larval sizes and underutilized yolk sacs as the copper concentrations get higher.

Table 3 Nautilus, Fife 7-day Survival & Growth Test Results with Three Reference Toxicants

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
NEFlarv01	4/5/2010	Copper chloride	7-day Survival	359.7	434.1	200	400	20.5%
			Biomass	189.9	469.5	200	400	30.3%
			Weight	617.6	> 800	400	800	30.2%
NEFlarv02	4/12/2010	Copper chloride	7-day Survival	399.3	552.8	200	400	23.5%
			Biomass	245.8	490.8	200	400	30.4%
			Weight	604.7	> 800	400	800	19.2%
NEFlarv03	5/17/2010	Copper chloride	7-day Survival	572.8	717.6	400	800	35.1%
			Biomass	448.4	630.2	400	800	35.9%
			Weight	694.6	> 800	400	800	20.0%
NEFlarv04	3/2/2011	Copper chloride	7-day Survival	NC	537.1	400	800	17.3%
			Biomass	131.4	467.0	100	200	18.3%
			Weight	294.9	780.0	200	400	18.7%
Coefficients of Variation			**7-day Survival**	**0.26**	**0.21**			
			Biomass	**0.54**	**0.15**			
			Weight	**0.32**				

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
NEFlarv05	4/6/2010	Potassium chloride	7-day Survival	567.8	617.8	250	500	11.6%
			Biomass	233.9	565.4	125	250	20.4%
			Weight	429.8	> 1000	250	500	22.3%
NEFlarv06	4/13/2010	Potassium chloride	7-day Survival	504.9	579.3	500	1000	24.8%
			Biomass	320.9	547.9	250	500	31.5%
			Weight	495.8	> 500	250	500	20.5%
NEFlarv07	5/18/2010	Potassium chloride	7-day Survival	488.3	531.1	250	500	15.6%
			Biomass	254.5	482.4	250	500	21.8%
			Weight	> 500	> 500	250	500	14.5%
NEFlarv08	5/18/2010	Potassium chloride	7-day Survival	560.8	594.6	500	1000	15.3%
			Biomass	206.4	420.3	125	250	16.4%
			Weight	246.0	590.7	125	250	14.0%
Coefficients of Variation			**7-day Survival**	**0.07**	**0.06**			
			Biomass	**0.19**	**0.13**			
			Weight	**0.33**				

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
NEFlarv09	4/14/2010	Sodium dodecyl sulfate	7-day Survival	1.43	1.75	1.25	2.5	13.8%
			Biomass	1.46	1.76	1.25	2.5	26.8%
			Weight	> 1.25	> 1.25	1.25	> 1.25	19.1%
NEFlarv10	5/19/2010	Sodium dodecyl sulfate	7-day Survival	NC	0.87	0.625	1.25	19.0%
			Biomass	0.35	0.71	< 0.625	0.625	32.4%
			Weight	0.38	> 0.625	< 0.625	0.625	27.7%
NEFlarv11	5/19/2010	Sodium dodecyl sulfate	7-day Survival	NC	0.88	0.625	1.25	18.4%
			Biomass	0.76	0.91	0.625	1.25	29.2%
			Weight	> 0.625	> 0.625	0.625	> 0.625	26.7%
NEFlarv12	5/20/2010	Sodium dodecyl sulfate	7-day Survival	NC	0.88	0.625	1.25	8.2%
			Biomass	0.63	0.81	0.3125	0.625	21.7%
			Weight	0.53	> 0.625	0.3125	0.625	19.0%
Coefficients of Variation			**7-day Survival**		**0.40**			
			Biomass	**0.59**	**0.46**			
			Weight	**0.24**				

Table 4. Shannon Point, Nautilus, Burnaby, and New Fields 7-day Survival & Growth Test Results with Copper

Shannon Point Marine Center

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
SPMClarv01	3/8/2010	Copper chloride	7-day Survival	NC	743.2	540	900	16.0%
			Biomass	563.8	683.9	540	900	17.1%
			Weight	691.9	> 900	540	900	19.2%
SPMClarv02	4/6/2010	Copper chloride	7-day Survival	194.6	445.5	< 117	117	11.8%
			Biomass	52.9	261.5	< 117	117	23.2%
			Weight	174.7	> 540	117	194	23.8%
SPMClarv03	4/14/2010	Copper chloride	7-day Survival	170.5	283.2	117	194	11.3%
			Biomass	76.4	394.4	324	540	25.0%
			Weight	429.0	> 540	324	540	25.8%
SPMClarv04	2/9/2011	Copper chloride	7-day Survival	536.2	NC	324	540	10.1%
			Biomass	371.9	540.0	324	540	17.2%
			Weight	471.2	> 540	324	540	18.7%
Coefficients of Variation			**7-day Survival**	**0.68**	**0.48**			
			Biomass	**0.92**	**0.39**			
			Weight	**0.48**				

Nautilus Environmental, Burnaby

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
NEBlarv01	4/13/2010	Copper chloride	7-day Survival	294.1	427.1	324	540	14.1%
			Biomass	367.9	433.1	324	540	28.1%
			Weight	> 540	> 540	540	> 540	31.2%
NEBlarv02	4/13/2010	Copper chloride	7-day Survival	390.6	445.3	324	540	15.8%
			Biomass	333.1	418.2	324	540	21.7%
			Weight	407.4	> 540	324	540	17.2%
NEBlarv03	4/14/2010	Copper chloride	7-day Survival	269.5	352.3	194	324	19.0%
			Biomass	202.9	290.1	194	324	22.0%
			Weight	277.3	470.8	194	324	25.5%
NEBlarv04	4/14/2010	Copper chloride	7-day Survival	323.8	399.7	194	324	27.8%
			Biomass	252.9	390.6	324	540	37.1%
			Weight	486.5	> 540	540	> 540	30.7%
Coefficients of Variation			**7-day Survival**	**0.16**	**0.10**			
			Biomass	**0.26**	**0.17**			
			Weight	**0.27**				

New Fields

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
NFlarv01	4/12/2010	Copper chloride	7-day Survival	138.2	277.0	324	540	18.9%
			Biomass	17.1	142.5	194	324	27.3%
			Weight	135.4	> 540	194	324	26.3%
NFlarv02	2/11/2011	Copper chloride	7-day Survival	359.5	489.3	200	400	23.5%
			Biomass	113.0	381.2	200	400	30.4%
			Weight	567.1	> 900	400	800	19.2%
NFlarv03	2/11/2011	Copper chloride	7-day Survival	299.8	449.6	194	324	14.9%
			Biomass	113.4	338.4	194	324	24.2%
			Weight	246.9	> 900	324	540	28.9%
NFlarv04	2/15/2011	Copper chloride	7-day Survival	407.6	537.9	324	540	20.3%
			Biomass	244.5	390.5	194	324	24.1%
			Weight	293.1	617.5	194	324	17.5%
Coefficients of Variation			**7-day Survival**	**0.39**	**0.26**			
			Biomass	**0.77**	**0.37**			
			Weight	**0.59**				

Embryo survival and development test

The herring embryo survival & development test results with all three reference toxicants from the Nautilus Environmental lab in Fife are shown in Table 5. These results show:

- All of the CVs for the Nautilus, Fife embryo survival & development tests were under 40% and easily met the goal of being less than 60%.
- 12 of the 13 PMSDs for live hatch were under 40%. The one PMSD for live hatch which exceeded 40% was 54.5%. 11 of the 13 PMSDs for normal survival were under 40%. The two PMSDs for normal survival which exceeded 40% were 41.3% and 61.2%.

Table 5. Nautilus, Fife embryo survival & development test reference toxicant results

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates		Control Comparisons		
				EC25	EC50	NOEC	LOEC	PMSD
NEFemb01	3/4/2005	Copper chloride	Live Hatchout	264.0	315.6	200	400	29.0%
			Normal Survival	250.0	295.5	200	400	29.9%
NEFemb02	3/18/2005	Copper chloride	Live Hatchout	276.0	381.4	150	300	20.5%
			Normal Survival	234.8	310.6	150	300	20.6%
NEFemb03	5/4/2005	Copper chloride	Live Hatchout	138.2	225.7	100	200	20.9%
			Normal Survival	111.4	187.2	50	100	19.0%
NEFemb04	5/13/2005	Copper chloride	Live Hatchout	371.5	383.7	200	400	15.6%
			Normal Survival	246.9	324.4	200	400	17.2%
		Coefficients of Variation	**Live Hatchout**	**0.36**	**0.23**			
			Normal Survival	**0.32**	**0.22**			

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates		Control Comparisons		
				EC25	EC50	NOEC	LOEC	PMSD
NEFemb05	3/4/2005	Potassium chloride	Live Hatchout	NC	648.1	500	1000	13.9%
			Normal Survival	NC	633.7	500	1000	14.4%
NEFemb06	3/18/2005	Potassium chloride	Live Hatchout	586.1	581.8	500	1000	36.9%
			Normal Survival	548.6	536.5	500	1000	41.3%
NEFemb07	5/4/2005	Potassium chloride	Live Hatchout	525.3	636.1	500	1000	35.4%
			Normal Survival	503.6	595.0	500	1000	34.9%
NEFemb08	5/13/2005	Potassium chloride	Live Hatchout	590.8	670.0	500	1000	14.0%
			Normal Survival	537.1	655.6	500	1000	14.8%
		Coefficients of Variation	**Live Hatchout**	**0.06**	**0.06**			
			Normal Survival	**0.04**	**0.09**			

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates		Control Comparisons		
				EC25	EC50	NOEC	LOEC	PMSD
NEFemb09	3/4/2005	Sodium dodecyl sulfate	Live Hatchout	> 5	> 5	5	> 5	14.8%
			Normal Survival	> 5	> 5	5	> 5	17.0%
NEFemb10	3/18/2005	Sodium dodecyl sulfate	Live Hatchout	6.1	7.1	5	10	25.2%
			Normal Survival	6.0	7.0	5	10	26.8%
NEFemb11	5/4/2005	Sodium dodecyl sulfate	Live Hatchout	2.6	2.8	2.5	5	19.9%
			Normal Survival	2.6	2.8	2.5	5	18.7%
NEFemb12	5/13/2005	Sodium dodecyl sulfate	Live Hatchout	5.2	5.4	5	10	21.4%
			Normal Survival	5.2	5.4	5	10	26.7%
NEFemb13	5/27/2005	Sodium dodecyl sulfate	Live Hatchout	5.1	6.0	5	10	54.5%
			Normal Survival	NC	6.4	5	10	61.2%
		Coefficients of Variation	**Live Hatchout**	**0.31**	**0.34**			
			Normal Survival	**0.38**	**0.34**			

The herring embryo survival & development test results with all three reference toxicants from the Shannon Point Marine Center are shown in Table 6. These results show:

- All of the CVs for the 25% effect and 50% effect point estimates from the Shannon Point Marine Center embryo survival & development tests were less than or equal to 40% and easily met the goal of being less than 60%.
- All of the 12 PMSDs for live hatch were under 40%. 11 of the 12 PMSDs for normal survival were under 40%. The one PMSD for normal survival which exceeded 40% was 47.6%.

Table 6. Shannon Point embryo survival & development test reference toxicant results

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
SPMCemb01	3/14/2002	Copper chloride	Live Hatchout	> 1000	> 1000	1000	> 1000	10.7%
			Normal Survival	377.2	516.0	316	422	15.6%
SPMCemb02	2/6/2003	Copper chloride	Live Hatchout	693.2	917.6	700	> 700	32.6%
			Normal Survival	288.5	338.7	221	295	23.1%
SPMCemb03	3/24/2003	Copper chloride	Live Hatchout	351.1	696.4	< 190	190	6.3%
			Normal Survival	179.7	249.9	< 190	190	5.7%
SPMCemb04	3/25/2003	Copper chloride	Live Hatchout	529.3	629.7	450	600	10.3%
			Normal Survival	162.2	227.5	< 190	190	9.6%
		Coefficients of Variation	**Live Hatchout**	**0.33**	**0.20**			
			Normal Survival	**0.40**	**0.39**			

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
SPMCemb05	1/29/2002	Potassium chloride	Live Hatchout	> 1200	> 1200	1200	> 1200	14.5%
			Normal Survival	444.7	474.2	380	506	47.6%
SPMCemb06	3/24/2003	Potassium chloride	Live Hatchout	699.8	715.3	600	800	6.5%
			Normal Survival	592.3	617.9	450	600	9.9%
SPMCemb07	3/24/2003	Potassium chloride	Live Hatchout	685.5	719.9	600	800	6.0%
			Normal Survival	494.0	531.7	450	600	7.0%
SPMCemb08	3/25/2003	Potassium chloride	Live Hatchout	674.1	721.2	450	600	8.1%
			Normal Survival	511.5	551.0	450	600	12.6%
		Coefficients of Variation	**Live Hatchout**	**0.19**	**0.004**			
			Normal Survival	**0.12**	**0.11**			

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates EC25	Point Estimates EC50	Control Comparisons NOEC	Control Comparisons LOEC	Control Comparisons PMSD
SPMCemb09	3/13/2002	Sodium dodecyl sulfate	Live Hatchout	2.0	2.2	1.41	1.88	10.3%
			Normal Survival	1.9	1.9	0.79	1.05	14.3%
SPMCemb10	1/25/2003	Sodium dodecyl sulfate	Live Hatchout	1.9	2.1	1.8	2.4	15.6%
			Normal Survival	1.6	1.8	1.01	1.35	16.9%
SPMCemb11	2/5/2003	Sodium dodecyl sulfate	Live Hatchout		2.3	1.8	2.4	20.3%
			Normal Survival		2.1	1.8	2.4	26.4%
SPMCemb12	3/25/2003	Sodium dodecyl sulfate	Live Hatchout	1.0	1.2	0.76	1.01	9.1%
			Normal Survival	0.9	1.0	0.76	1.01	6.3%
		Coefficients of Variation	**Live Hatchout**	**0.34**	**0.26**			
			Normal Survival	**0.36**	**0.29**			

The herring embryo survival & development test results with copper chloride from the Nautilus Environmental lab in Burnaby are shown in Table 7. These results show:

- One of the CVs for the Nautilus, Burnaby embryo survival & development tests was over 60%. The CV for the live hatch EC25 was 72%. The CVs for the normal survival EC25 and the live hatch and normal survival EC50s were 35% or less.
- All of the PMSDs for live hatch were under 40%. 4 of the 5 PMSDs for normal survival were under 40%. The one PMSD for normal survival which exceeded 40% was 41.0%.

Table 7. Nautilus, Burnaby Embryo Survival & Development Test Copper Results

Test Code	Start Date	Reference Toxicant	Biological Endpoint	Point Estimates		Control Comparisons		
				EC25	EC50	NOEC	LOEC	PMSD
NEBemb01	3/19/2010	Copper chloride	Live Hatchout	91.7	340.3	100	200	29.2%
			Normal Survival	178.6	195.7	100	200	41.0%
NEBemb02	4/15/2010	Copper chloride	Live Hatchout	137.9	265.7	200	400	15.4%
			Normal Survival	117.4	193.1	50	100	19.5%
NEBemb03	4/15/2010	Copper chloride	Live Hatchout	113.7	247.9	50	100	28.5%
			Normal Survival	98.6	157.8	100	200	34.4%
NEBemb04	3/17/2011	Copper chloride	Live Hatchout	340.2	498.9	200	400	29.2%
			Normal Survival	244.5	285.1	200	400	26.6%
NEBemb05	3/18/2011	Copper chloride	Live Hatchout	70.0	233.5	< 50	50	25.0%
			Normal Survival	183.1	241.9	100	200	26.5%
		Coefficients of Variation	**Live Hatchout**	**0.72**	**0.35**			
			Normal Survival	**0.35**	**0.23**			

Overall Results

- The Shannon Point Marine Center, Nautilus, Fife, Nautilus, Burnaby, and New Fields labs together conducted 16 copper chloride reference toxicant tests using the larval herring 7-day survival & growth test method. The CVs from the LC25s and LC50s were 37% and 28% respectively. The CVs from the biomass IC25s and IC50s were 66% and 32% respectively. The CVs from the weight IC25s and IC50s were 43% and 25% respectively.
- The Shannon Point Marine Center, Nautilus, Fife, and Nautilus, Burnaby labs together conducted 13 copper chloride reference toxicant tests using the herring embryo survival & development test. The CV from live hatch EC25s from all three labs was 68%. The CV from live hatch EC50s was 51%. The CVs from normal survival EC25s and EC50s were 38% and 34% respectively.
- The mean PMSDs from all labs for all test materials were below 30%. The mean PMSD for 96-hour survival was 14.5%. The mean PMSDs from the 7-day survival & growth test were 17.8% for 7-day survival, 25.5% for biomass, and 22.3% for weight. The mean PMSDs from the embryo survival & development test were 20.0% for live hatch and 22.9% for normal survival.
- Appendix B contains the concentration-response graphs for all herring test results in our CETIS database. The concentration-response relationships generally look good and resemble the responses of standard toxicity test species in the CETIS database. These graphs are organized by test type and test code in the same way as Tables 1 – 7. The test

materials include reference toxicants, effluents, creosote leachate, and ambient water samples from the Cherry Point Reach.

Conclusions

The ten year effort to develop and validate herring toxicity tests was successful. We now have three herring toxicity tests that are well-established locally and have already had some limited regulatory use for monitoring effluents and assessing ballast water biocide toxicity. However, the use of the herring toxicity tests for monitoring effluents is constrained by the availability of test organisms during spawning season. Due to this constraint, the herring tests should only be used for screening effluents for toxicity to herring, and the full regulatory application should be reserved for the standard toxicity tests which are readily available all year. Herring are a key regional species and screening for risks is important. Testing of environmental samples is another very important need for the herring toxicity tests. More details supporting the conclusions that the herring tests are both ready and needed are in the lists below.

1. The herring tests are ready for use:

    - The herring tests have already established a track record of use for reference toxicant testing, effluent monitoring, testing environmental samples, evaluating ballast water biocides, examining creosote toxicity, assessing dinoflagellate toxicity, and comparing the embryo temperature tolerance of different West Coast herring stocks (See Appendix C.).
    - The three herring tests demonstrated the ability to detect differences in response of 40% or greater as statistically significant. The overall PMSD averages for each test were lower than 30%. Occasional PMSDs were above 40%, but our CETIS database shows that to also be the case for the standard EPA tests. All of the labs individually demonstrated the ability to achieve PMSDs below 40% in most of their herring test results.
    - Only the 25% effect level point estimates produced an occasional CV over 60%. CVs from the 50% effect level point estimates always met the less than 60% criterion. This fact shows that much of the variability contributing to higher CVs comes from the point estimate calculation at the 25% effect level and not from test organism performance.
    - The only interlaboratory CV which failed for any of the endpoints from the three herring tests was the CV for the live hatch EC25s. The live hatch EC50s and the EC25s for all of the other larval and embryo endpoints provided interlaboratory CVs below 60%.
    - The Nautilus Environmental lab in Fife successfully validated all three herring tests using all three reference toxicants. The Shannon Point Marine Center successfully validated the herring embryo survival & development test using all three reference toxicants.
    - The herring tests can pass the validation criteria using all three reference toxicants. However, the use of only one reference toxicant is standard quality control for the established toxicity tests. Copper chloride is a popular reference toxicant. Potassium chloride and sodium dodecyl sulfate produced none of the CVs over 60%. Lab evaluations based upon copper chloride results are adequate without potassium chloride and sodium dodecyl sulfate results. It is also more economical to use one reference toxicant.
    - The Nautilus Environmental lab in Burnaby successfully validated the larval 7-day survival & growth test and the embryo survival & development test using copper

chloride. The CVs for the survival & growth test were especially good (all less than 30%). The CVs for the embryo survival & development test were all less than 40% except for the live hatch EC25 which had a CV of 72%.

- The New Fields lab in Port Gamble successfully validated the larval 7-day survival and growth test using copper chloride. The CV for the biomass IC25 exceeded 60% but this was due to one test result and the CV for the biomass IC50 was well under 60%. In addition, the NOEC – LOEC pairs were identical for three of the four tests and the remaining NOEC – LOEC pair was not much different. The NOEC and LOEC are a better representation of regulatory decisions in this state than the IC25.
- The Nautilus Environmental lab in Burnaby and the New Fields lab in Port Gamble can fill the gap from the closure of the Nautilus Environmental lab in Fife.
- The interlaboratory evaluation showed a problem with the consistency of live hatch as measured by the 25% effect level point estimate. In addition, the Nautilus Environmental lab in Burnaby had a CV over 60% for the live hatch EC25. Live hatch results should be excluded from sensitive regulatory decisions until copper chloride reference toxicant testing has met the validation criterion of a CV less than or equal to 60%. In addition to quality control plotting, the results of the last four routine copper chloride reference toxicant tests should have a CV for the live hatch EC25 that is less than 60% or only the normal survival results will be considered.

2. The herring tests are needed:

    - WDFW has reported locations within the spawning grounds of the Quartermaster Harbor, Port Gamble, and Port Orchard/Madison herring stocks where eggs usually die soon after deposition. The chemicals causing these mortalities remain unknown.
    - Dr. Richard Kocan and Dr. Paul Hershberger assessed Cherry Point spawning zone conditions by exposing herring embryos at 12 stations along the shoreline. The percentages of abnormal larvae from these outplants were averaged for the 4 years (1990, 1991, 1992, and 1998) of study. See Table 8. The average percent abnormal ranged from 54.3% at the worst station to 25.4% at the best station. Stations that are adjacent along the shoreline tend to also be adjacent in the table when ranked by percent abnormal. The probability that this pattern occurred due to chance alone is nearly 5000:1. The northern six stations had significantly ($\alpha = 0.05$) better development than the southern six stations. Even though the whole shoreline was once used, herring spawned during these years only near the northernmost stations. Testing to determine cause and effect has not yet been done.

Table 8. Abnormality rates for herring outplants at Cherry Point in the 1990s

location	stations numbered north to south	average % abnormal
s. of Al smelter pier	7	54.3
ravine	8	43.0
s. of oil refinery pier	10	40.8
n. of oil refinery pier	9	40.2
gravel pier	5	38.7
Neptune Beach	11	38.7
Sandy Point	12	35.1
n. of Al smelter pier	6	34.6
Viewpoint	2	30.8
n. of oil refinery pier	3	30.7
s. of oil refinery pier	4	27.8
Point Whitehorn	1	25.4
lab controls		29.4

- Herring early lifestages are sensitive to polycyclic aromatic hydrocarbons and to creosote. The herring tests would be good for assessing whether cleanup of these or other materials is adequate.
- Herring tests would be good for investigating the effects of harmful algal blooms, rising water temperatures, and increasing ocean acidification on fish early lifestages.

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References

Dinnel, P.A., D.P. Middaugh, N.T. Schwarck, H.M. Farren, R.K. Haley, R.A. Hoover, J. Elphick, K. Tobiason, R.R. Marshall. 2011. Methods for Conducting Bioassays Using Embryos and Larvae of Pacific Herring, *Clupea pallasi*. Arch Environ Contam Toxicol 60:290–308.

Dinnel, P.A., R. Hoover, L. Lechuga, K. Tobiason, J. Elphick. 2008. Development of Larval Pacific Herring, *Clupea pallasi*, Bioassay Protocols: Refinement, Validation, Refinery Effluent and Cherry Point Ambient Water Testing During 2007. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 58 pp.

Dinnel, P.A. 2008. Pacific herring, *Clupea pallasi*, embryo and larval bioassay protocols. Summary Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 18 pp.

Dinnel, P.A., C. Montanez, K. Bergmann, J. Elphick. 2007. Refinement of the larval Pacific herring, *Clupea pallasi*, survival and growth bioassay protocol. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 63 pp.

Dinnel, P.A., L. Paisano, A. Shi, J. Elphick, K. Bergmann, J. Alaimo. 2006. Development of embryo and larval Pacific herring, *Clupea pallasi*, Bioassay protocols: phase V. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 89 pp + appendix.

Dinnel, P.A., H.M. Farren, L. Marko, S.A. Morales. 2005. Development of embryo and larval Pacific herring, *Clupea pallasi*, bioassay protocols: phase IV. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 82 pp.

Dinnel, P.A., R.K. Haley, B. Keopaseut. 2003. Development of embryo and larval Pacific herring, *Clupea pallasi*, bioassay protocols: phase III. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 83 pp.

Dinnel, P.A., N.T. Schwarck, A. Balderas, M. Cotter, D.P. Middaugh. 2002. Development of embryo and larval Pacific herring, *Clupea pallasi*, bioassay protocols: phase II. Final report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA, 56 pp + appendices.

Hershberger, P.K., R.M. Kocan. 1999. Final Report – 1999, Survival potential of Cherry Point herring: larval abnormalities and weight at hatch following in situ incubation of developing embryos. Washington Department of Natural Resources #FY00-092.

Hershberger, P.K. and R.M. Kocan 2001. Final Report – 2000, Washington DNR herring study 2000: health of Puget Sound stocks. Washington Department of Natural Resources #FY00-183.

Marshall, R. 2008. Laboratory guidance and whole effluent toxicity test review criteria. Washington Department of Ecology Publ. No. WQ-R-95-80, December 2008 Revision, WDOE Water Quality Program, Olympia, WA, 78 pp

Stick, K. and A. Linquist. 2009. 2008 Washington State Herring Stock Status Report. Washington Department of Fish and Wildlife Stock Status Report No. FPA 05-09, November 2009.

Tidepool Scientific. 2010. CETIS (Comprehensive Environmental Toxicity Information System) v.1.8.0.4. Tidepool Scientific Software, McKinleyville, CA

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Appendix A. Glossary

biomass – The mass of the surviving organisms at the end of the test divided by the number of organisms at the beginning of the test. The biomass endpoint is contrasted with the weight endpoint which is the mass of surviving organisms at the end of the test divided by the number of organisms at the end of the test. The biomass endpoint accounts for biomass loss due to death.

CETIS (Comprehensive Environmental Toxicity Information System) – A database application produced by Tidepool Scientific Software (http://www.tidepool-scientific.com/) which includes the ability to perform a wide range of statistical analyses relevant for data from toxicity testing.

coefficient of variation (CV) – The standard deviation divided by the mean. The CV is used in this report as a measure of the variability of reference toxicant test results. Reference toxicant test results should ideally be nearly equal because the tests are run under identical controlled conditions. Higher CVs represent a higher "plus or minus factor" for the point estimates from the test results.

concentration-response relationship – Toxicity tests are conducted using a series of increasing concentrations of the toxicant with the expectation that adverse effects will increase as the toxicant concentration increases. Toxicity tests also include a nontoxic control to ensure that test organism performance meets minimum expectations and to use in statistical comparisons with the toxicant concentrations. The accuracy of some statistical analyses, such as linear interpolation, are dependent on adverse effects increasing incrementally as the toxicant concentration increases. Examination of the concentration-response relationship is also important in understanding test results such as the NOEC and LOEC. See Appendix B for examples of concentration-responses from this project.

EC25 and EC50 – "EC" stands for effect concentration. The EC25 is the toxicant concentration estimated to cause an adverse effect in 25% of the test organisms. The EC50 is the toxicant concentration estimated to cause an adverse effect in 50% of the test organisms. The "EC" is the generic designation for a point estimate and can be used for the other point estimates (IC25, IC50, LC25, and LC50) as well.

effect level – The degree of adverse effect in the test organisms relative to the control. For example, the LC25 represents 25% mortality or 75% survival. A biomass IC25 represents a 25% reduction in biomass relative to the control.

embryo – The earliest fish lifestage beginning with the first cell division after fertilization and continuing until hatch. The embryo is sometimes called the egg, but an egg also includes yolk and other egg structures such as the chorion which are not a part of the embryo.

larva – The lifestage beginning after the complete utilization of the yolk and the commencement of feeding until the larvae metamorphosis into juveniles. Herring larvae are poor swimmers and generally drift like plankton. Herring juveniles can swim against currents and go where they want to go.

IC25 and IC50 – "IC" stands for inhibition concentration. The IC25 is the toxicant concentration which inhibits biomass or weight by 25% relative to the control. The IC50

is the toxicant concentration which inhibits biomass or weight by 50% relative to the control.

LC25 and LC50 – "LC" stands for lethal concentration. The LC25 is the toxicant concentration estimated to cause 25% mortality. The LC50 is the toxicant concentration estimated to cause 50% mortality. The LC25 and LC50 are the same as the EC25 and EC50 applied specifically to lethality.

linear interpolation - An analysis whereby a curve is fit to nonquantal concentration-response data so that point estimates at specific effect levels can be made. Although linear interpolation can be performed on quantal data, it is usually reserved for nonquantal weight and reproduction data. In order to be accurate, linear interpolation depends on adverse effects increasing incrementally as the toxicant concentration increases. Incremental increases in adverse effects do not always happen especially when the lower toxicant concentrations are below the toxic threshold and either chance or toxicant stimulation (hormesis) increase test organism survival, growth, or reproduction relative to the control.

linear regression – An analysis whereby an equation is fit to quantal concentration-response data using a model so that point estimates at specific effect levels can be made. The specific linear regression analysis used in the context of this project fits data to the probit model. If the data do not fit the model or if the concentration-response is too sharp (one or fewer partial responses), then linear regression will not run. Linear regression is the preferred analysis for estimating 25% effect levels from quantal data.

live hatch - The number of live herring after hatch divided by the number of eggs (embryos) at the beginning of the test.

LOEC (lowest observed effects concentration) – The LOEC is the lowest concentration in a toxicity test which shows a statistically significant difference from the control.

mean – The mean is the arithmetic average and is often just called the average. For the purposes of this report, the mean is calculated by adding all of the measurements and dividing by the number of measurements.

median effect level – The median effect level is the toxicant or sample concentration associated with a 50% effect on test organisms. Because the median effect level is determined from the midpoint (median) of a concentration-response curve, it is the best single representative for the overall test result. The median effect level has the highest precision of all effect levels and the narrowest confidence interval. For these reasons, the median effect level is usually preferred in comparative toxicology.

NOEC (no observed effects level) – The NOEC is the highest concentration in a toxicity test which does not show a statistically significant difference from the control. The NOEC is by definition the next concentration below the LOEC in the concentration series.

normal survival – The number of normal herring after hatch divided by the number of eggs (embryos) at the beginning of the test.

Pacific herring (*Clupea pallasi*) – Pacific herring are a key forage fish species in the region. They are an important link in the food chain process for converting zooplankton biomass into the biomass of larger fish such as salmon. From Stick et al, 2009:

Forage fishes in general, and herring specifically, are vital components of the marine ecosystem and are a valuable indicator of the overall health of the marine environment. Many species of sea birds, marine mammals, and finfish, including lingcod (*Ophiodon elongatus*), chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon, depend on herring as an important prey item.

PMSD (percent minimum significant difference) – Given the number of replicates used in a test and the variability in test organism response across those replicates, the PMSD is an estimate of the minimum difference needed between a test concentration and a control in order for that difference to be considered significantly different when subjected to a statistical comparison.

point estimation – Using toxicity test data to estimate a toxicant concentration that would cause a specified effect level. See **EC25 and EC50**, **IC25 and IC50**, **LC25 and LC50**, **linear interpolation**, **linear regression**, and **Spearman-Kärber**.

prolarva – Another common term for this lifestage is yolk-sac larva. These are larva which are still relying on the yolk sac for nourishment just after hatch and are relatively inactive. The 96-hour acute survival test is run using herring prolarva. The lower activity and interaction with the environment can make prolarvae less sensitive to some toxicants.

quantal and nonquantal data – Quantal results are derived by counting the number of organisms at the end of a test to get a number such as the number alive or the number normal which is then divided by the total number of organisms at the beginning of the test. A quantal result can therefore only occur as a number between 0 and 1 inclusive. Nonquantal results come from measuring a property of the test organisms such as weight or biomass and can be any number within the realm of possibility for the property being measured. Because quantal numbers are bounded by 0 and 1 and nonquantal numbers are not, different analyses are sometimes required. For example, linear regression and Spearman-Kärber only work with quantal data.

Spearman-Kärber – A nonparametric procedure for estimating the median effect level (LC50 or EC50) point estimate from quantal data. Because it is a nonparametric procedure, Spearman-Kärber will work with most toxicity test data showing a sufficiently large concentration-response. However, Spearman-Kärber only provides the 50% effect level.

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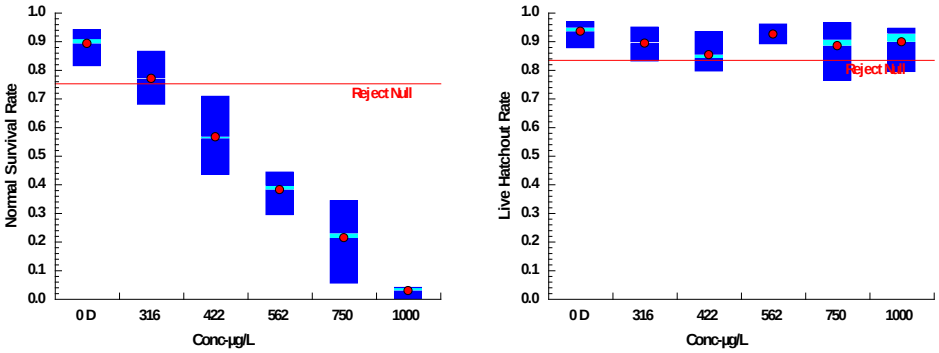
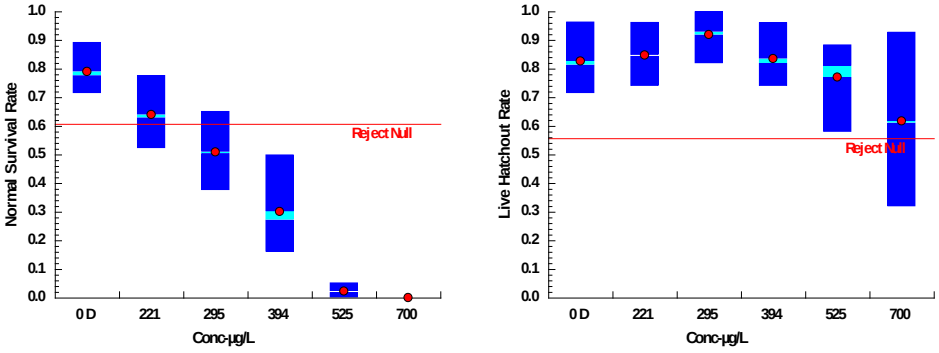
Appendix B. Concentration-Response Relationships for Embryo and Larval Tests

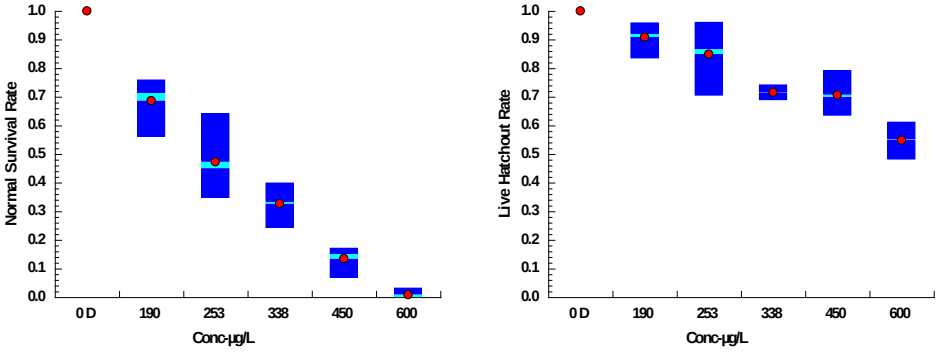
SPMCemb01 – 3/14/2002 – **$CuCl_2$**



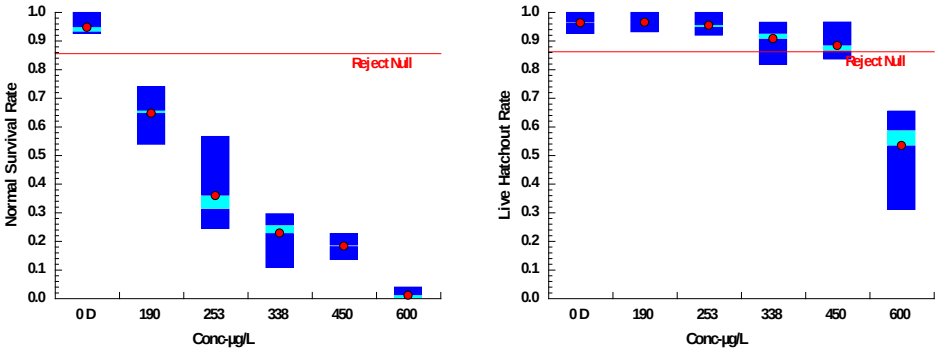
SPMCemb02 – 2/6/2003 – **$CuCl_2$**



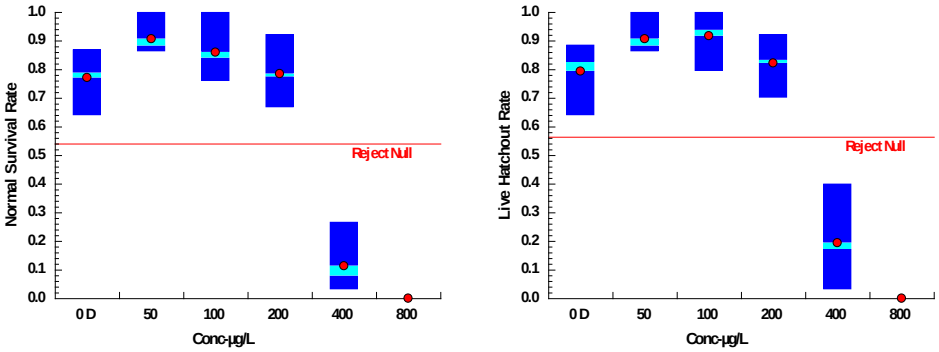
SPMCemb03 – 3/24/2003 – **$CuCl_2$**



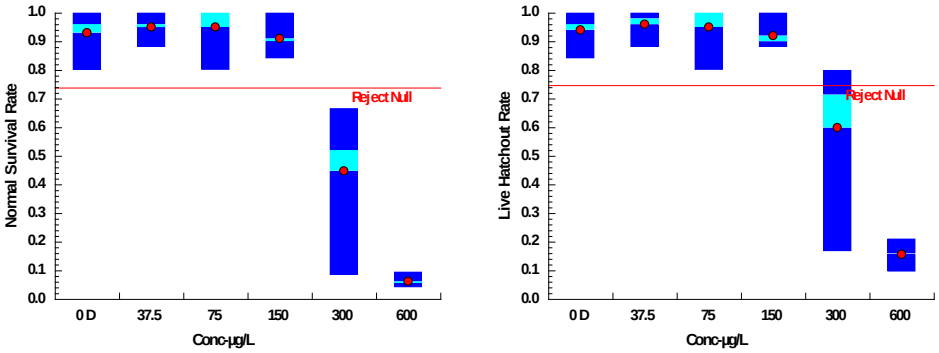
SPMCemb04 – 3/25/2003 – **$CuCl_2$**



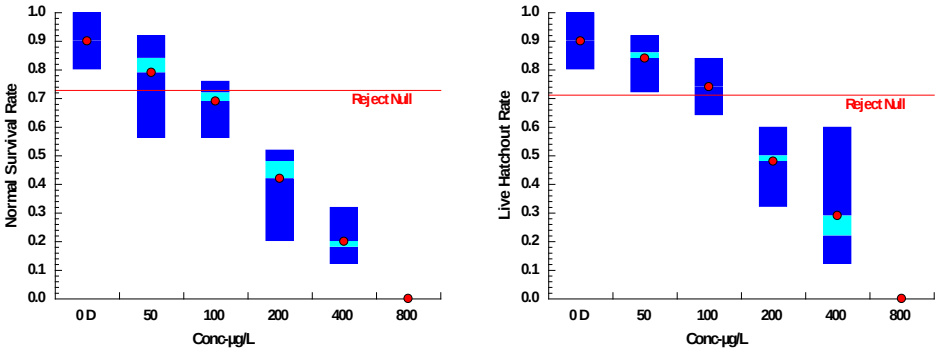
NEFemb01 – 3/4/2005 - **$CuCl_2$**



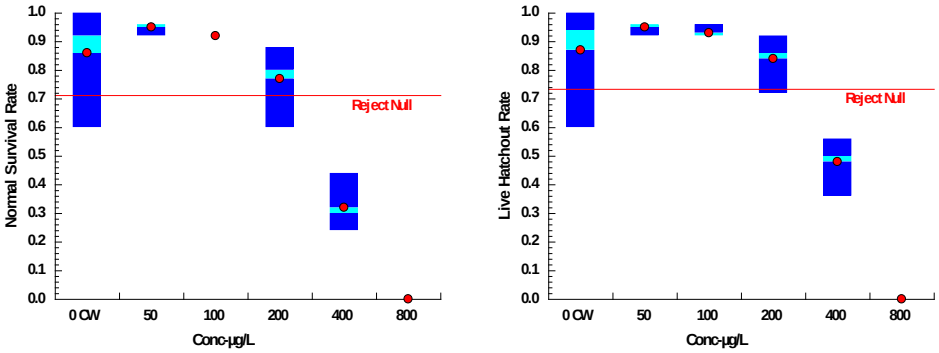
NEFemb02 – 3/18/2005 - **$CuCl_2$**



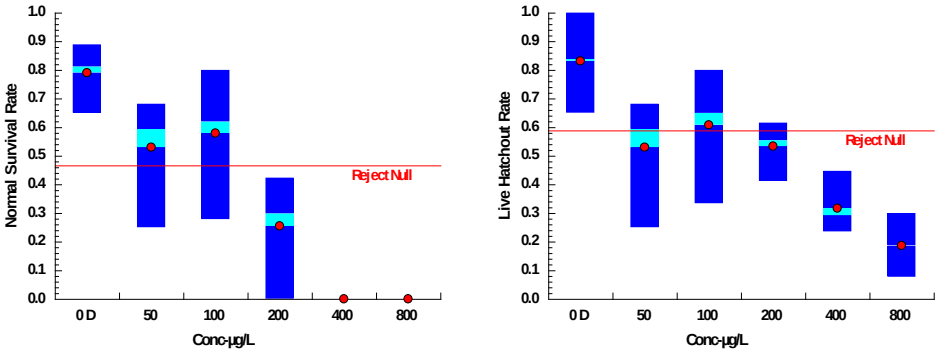
NEFemb03 – 5/4/2005 - **$CuCl_2$**



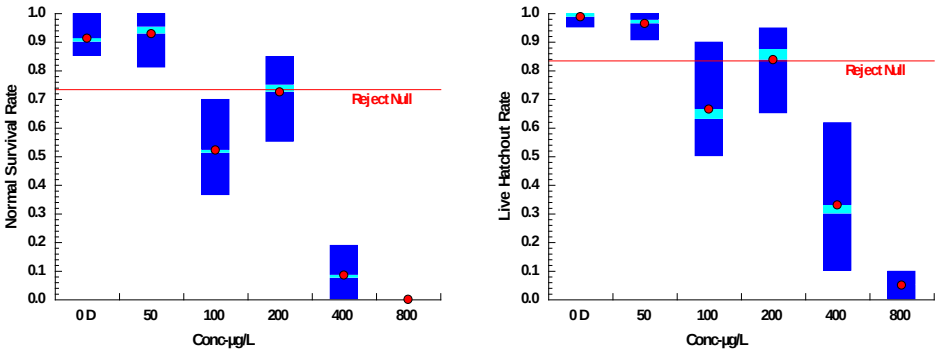
NEFemb04 – 5/13/2005 - **$CuCl_2$**



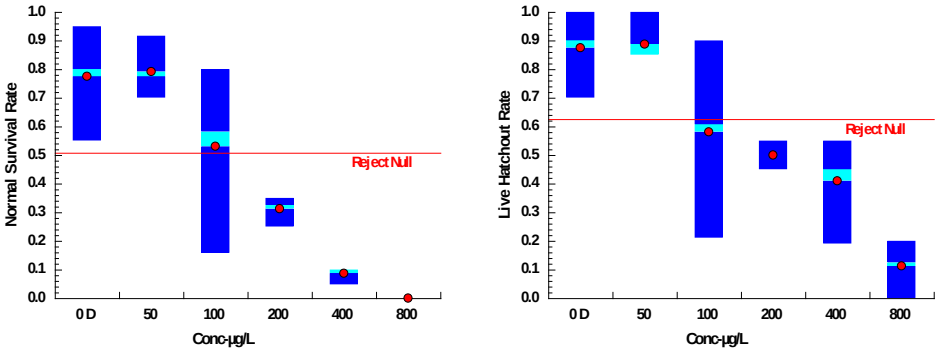
NEBemb01 – 3/19/2010 - **$CuCl_2$**



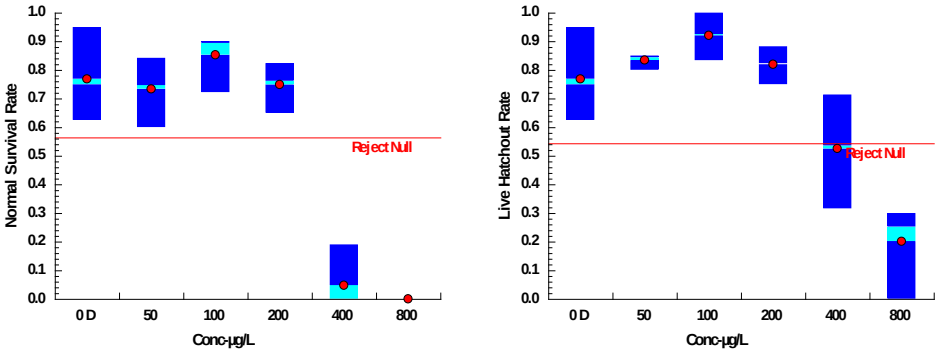
NEBemb02 – 4/15/2010 - **$CuCl_2$**



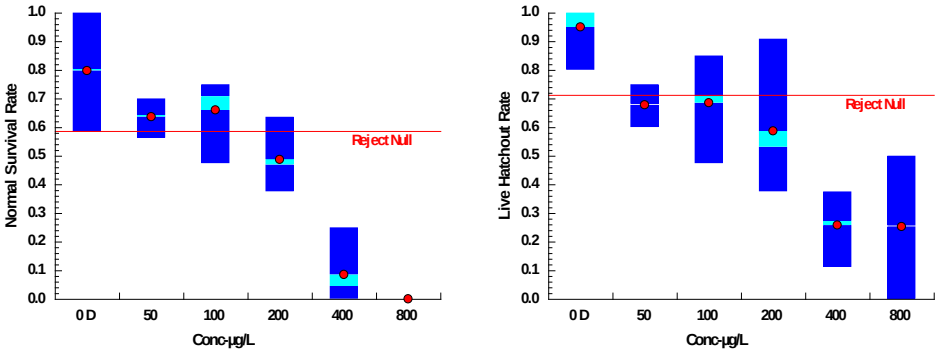
NEBemb03 – 4/15/2010 - **$CuCl_2$**



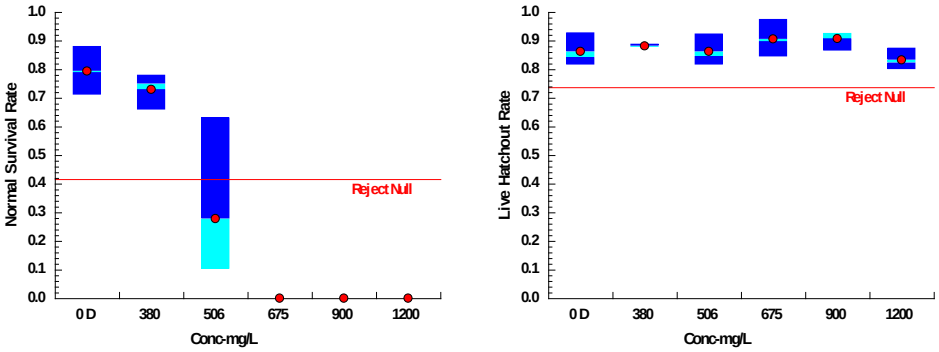
NEBemb04 – 3/17/2011 - **$CuCl_2$**



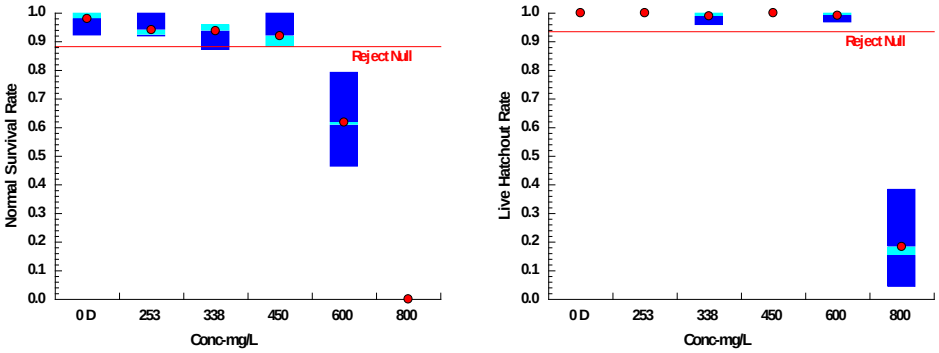
NEBemb05 – 3/18/2011 - **$CuCl_2$**



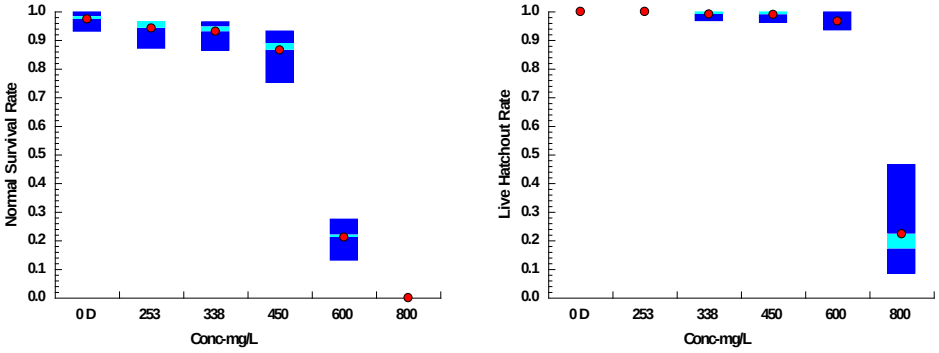
SPMCemb05 – 1/29/2002 – **KCl**



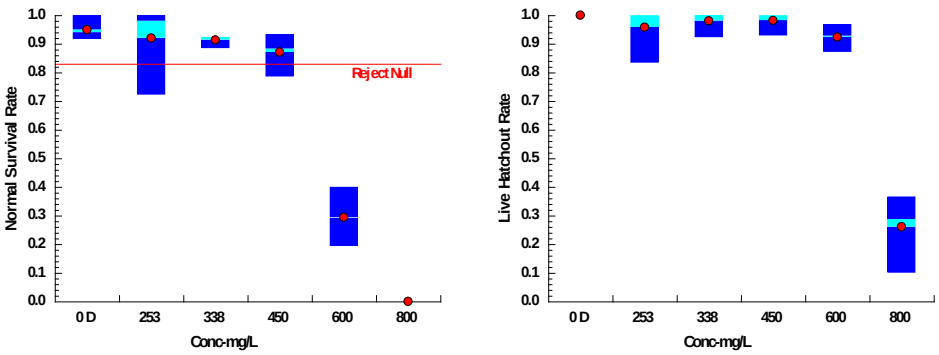
SPMCemb06 – 3/24/2003 – **KCl**



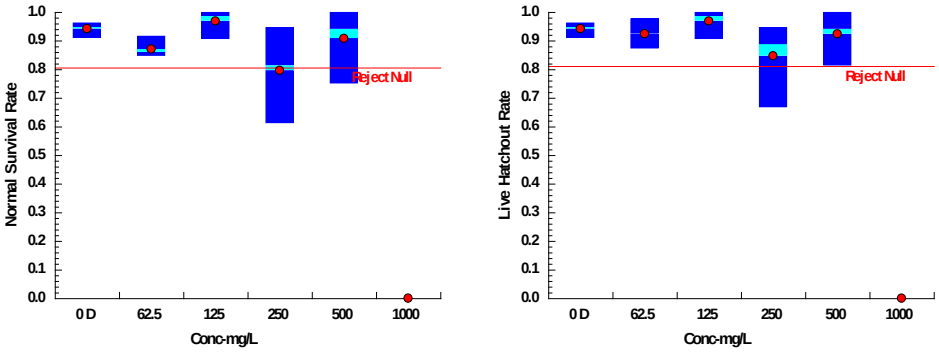
SPMCemb07 – 3/24/2003 – **KCl**



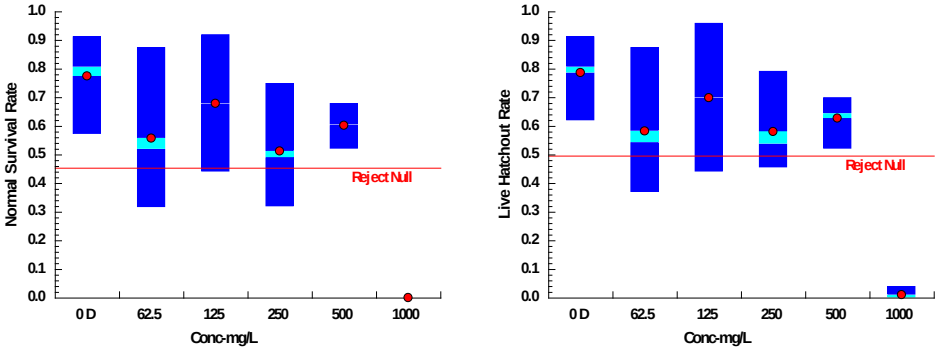
SPMCemb08 – 3/25/2003 – **KCl**



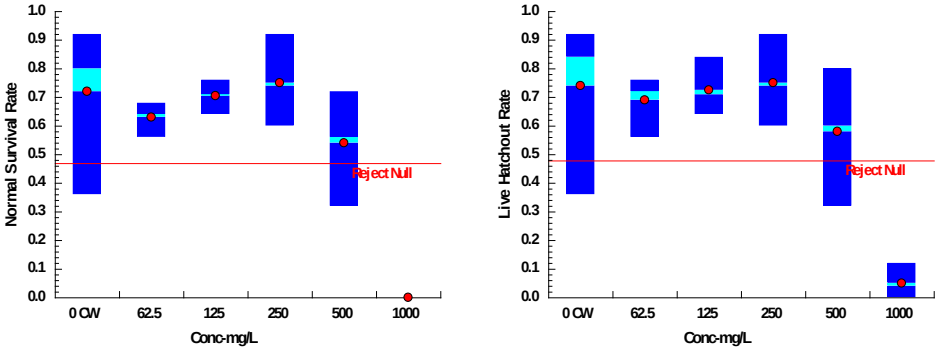
NEFemb05 – 3/4/2005 – **KCl**



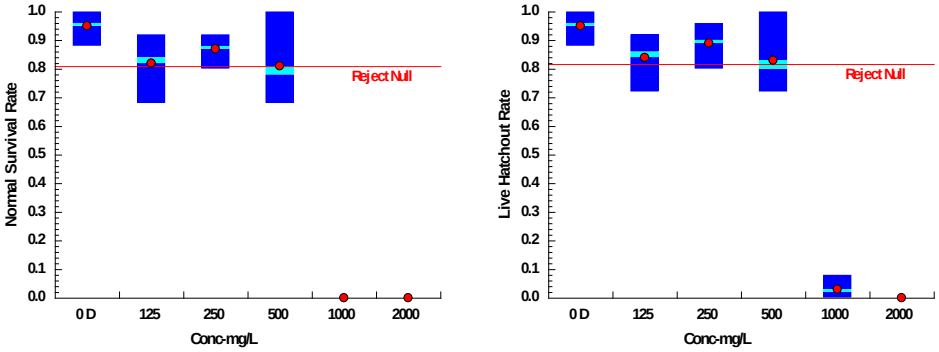
NEFemb06 – 3/18/2005 – **KCl**



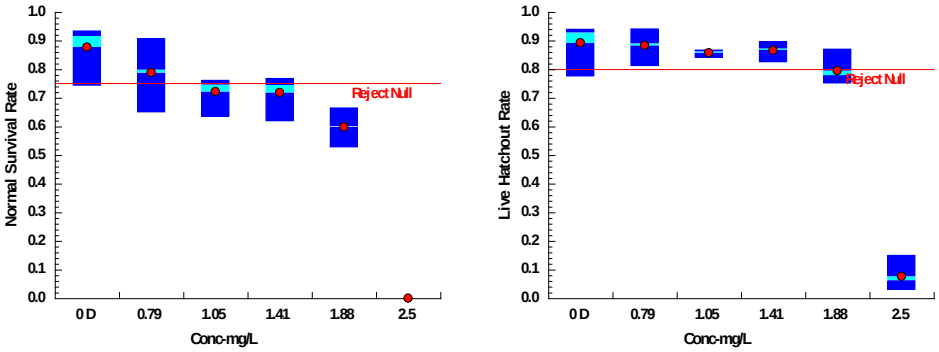
NEFemb07 – 5/4/2005 – **KCl**



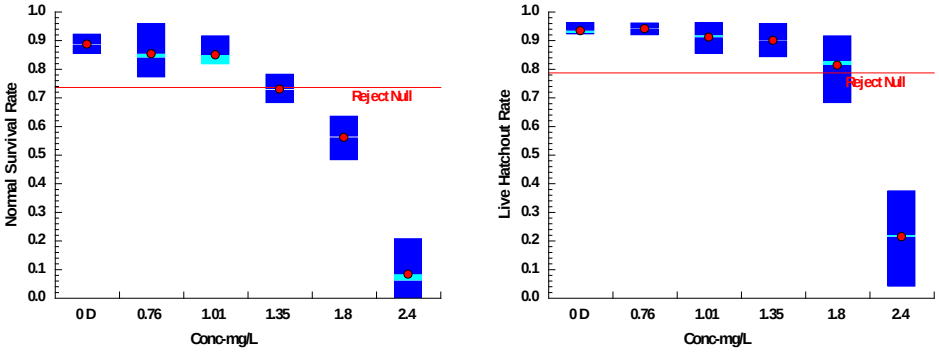
NEFemb08 – 5/13/2005 – **KCl**



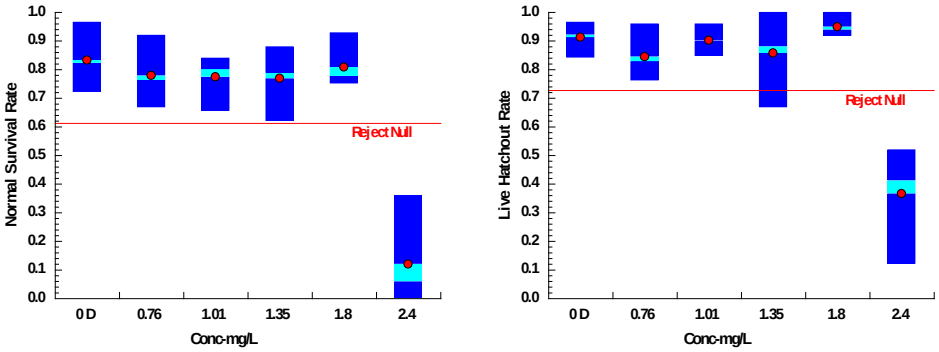
SPMCemb09 – 3/13/2002 – **SDS**



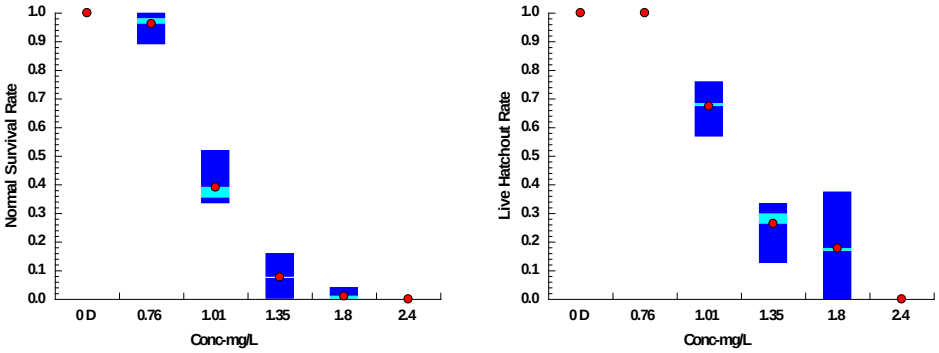
SPMCemb10 – 1/25/2003 – **SDS**



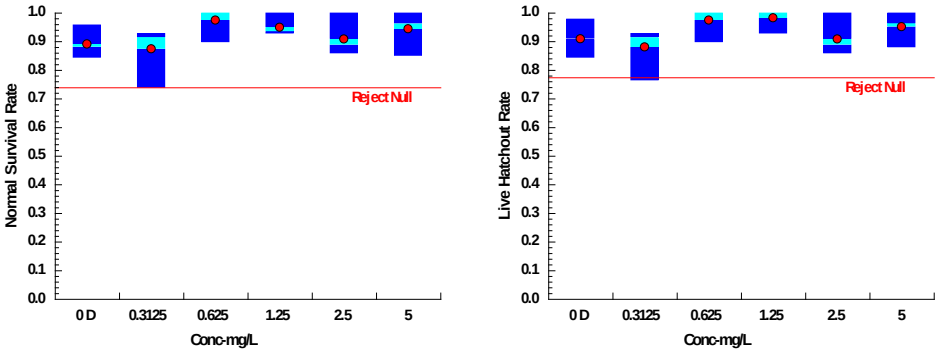
SPMCemb11 – 2/5/2003 – **SDS**



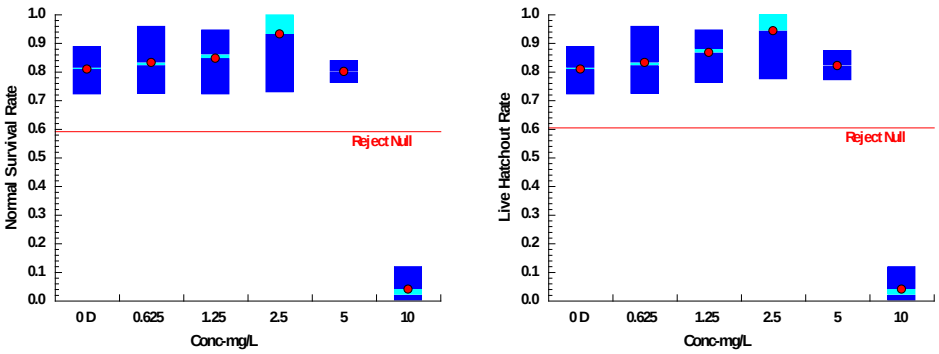
SPMCemb12 – 3/25/2003 – **SDS**



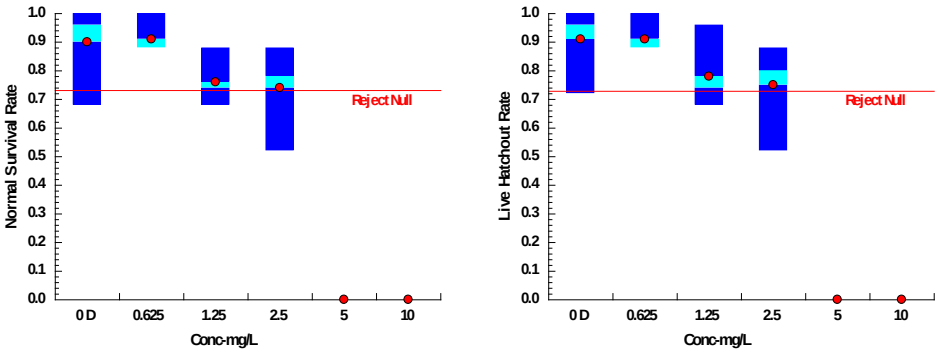
NEFemb09 – 3/4/2005 – **SDS**



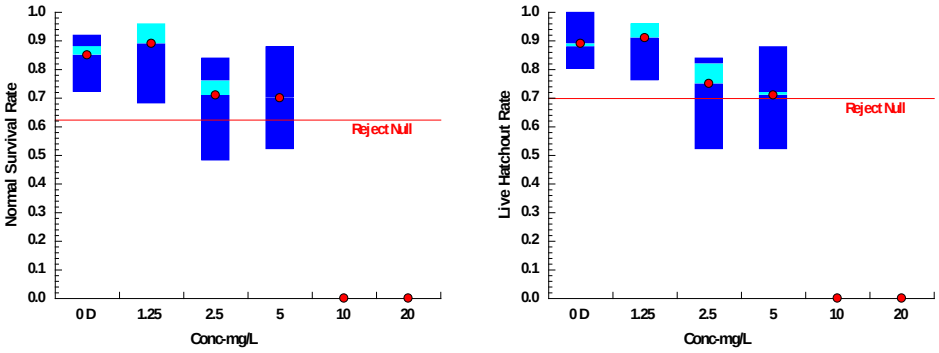
NEFemb10 – 3/18/2005 – **SDS**



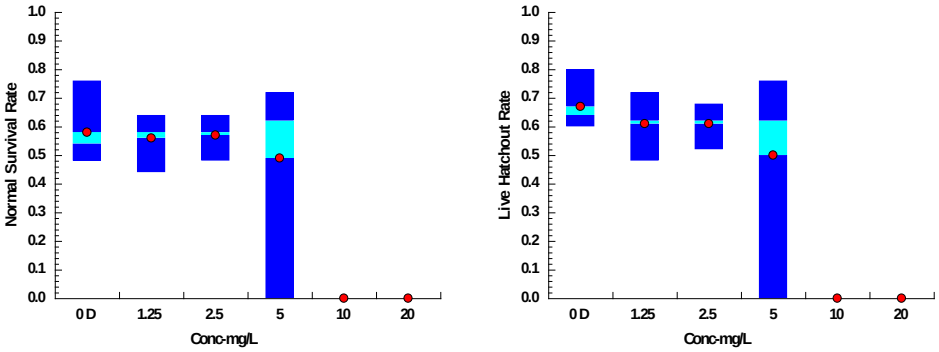
NEFemb11 – 5/4/2005 – **SDS**



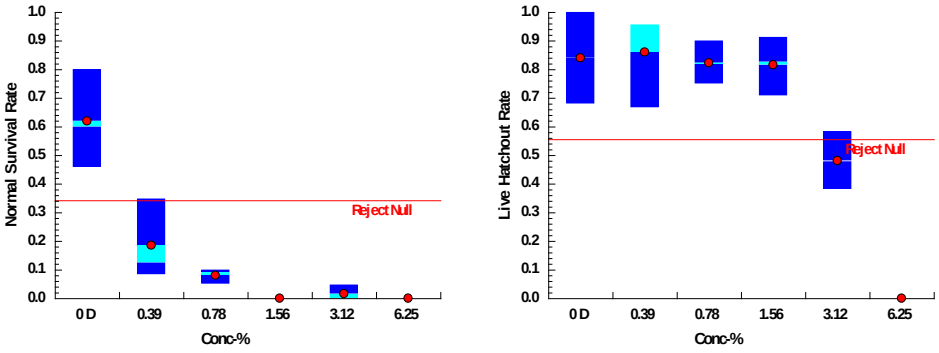
NEFemb12 – 5/13/2005 – **SDS**



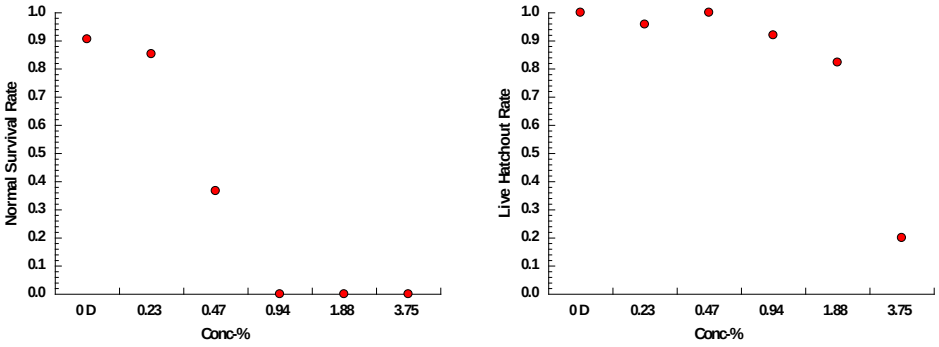
NEFemb13 – 5/27/2005 – **SDS**



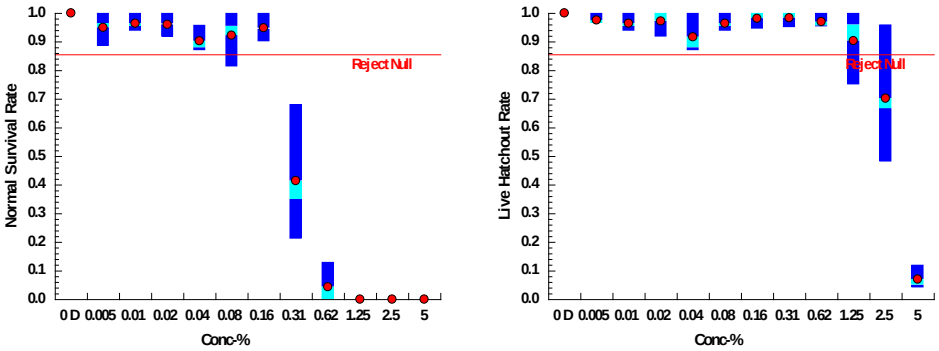
SPMCemb13 – 1/20/2005 - – % **creosote** saturated seawater



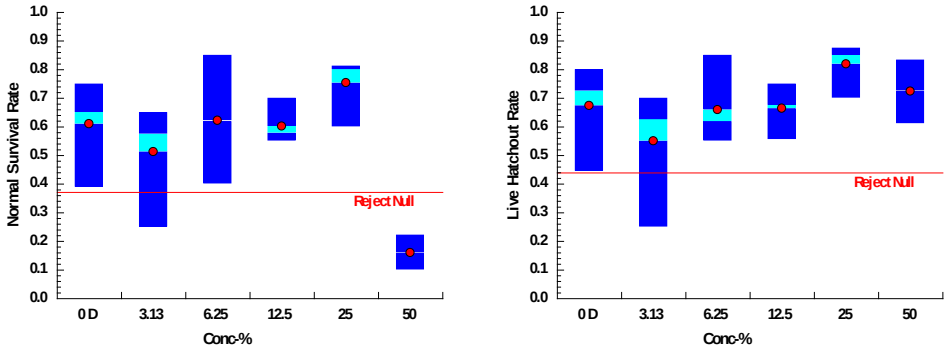
SPMCemb14 – 1/29/2005 - % **creosote** saturated seawater (insufficient replication required Fisher's Exact Test)



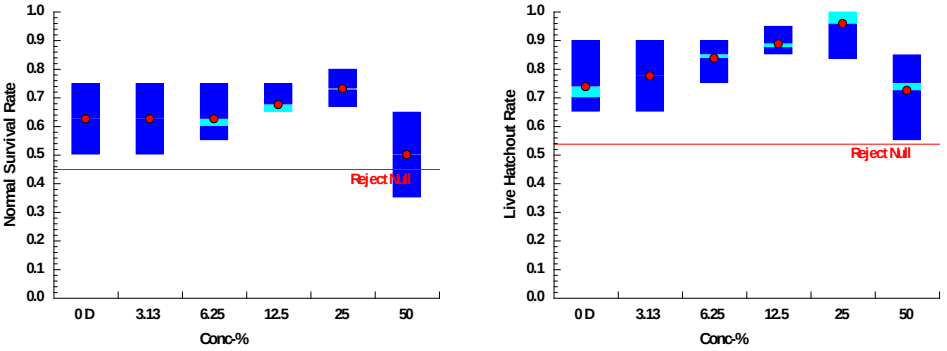
SPMCemb15 – 3/5/2005 – % **creosote** saturated seawater



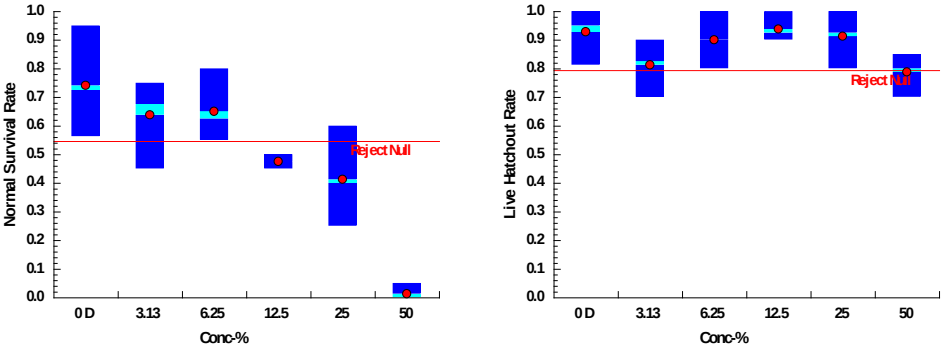
NEFemb14 – 5/17/2007 – **Oil Refinery 1 Effluent**



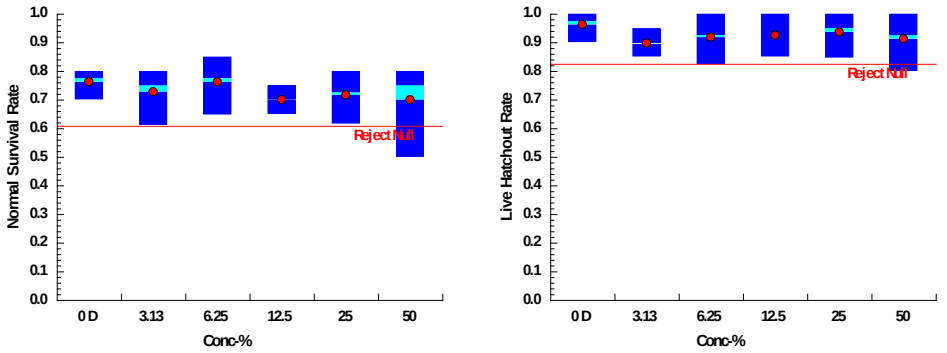
NEFemb15 – 5/16/2007 – **Oil Refinery 2 Effluent**



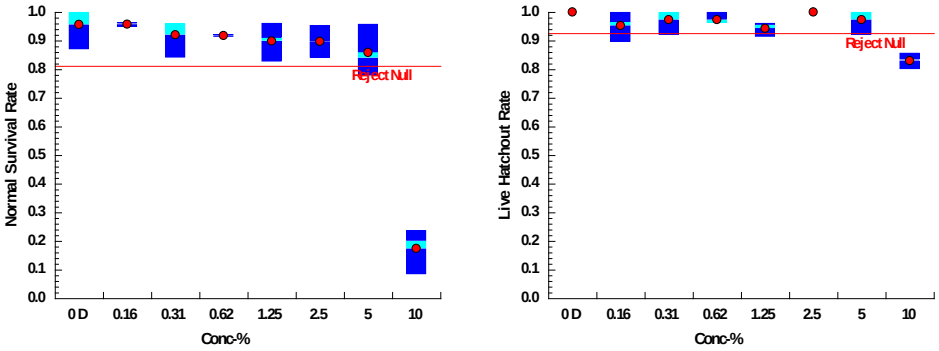
NEFemb16 – 5/16/2007 – **Oil Refinery 4 Effluent**



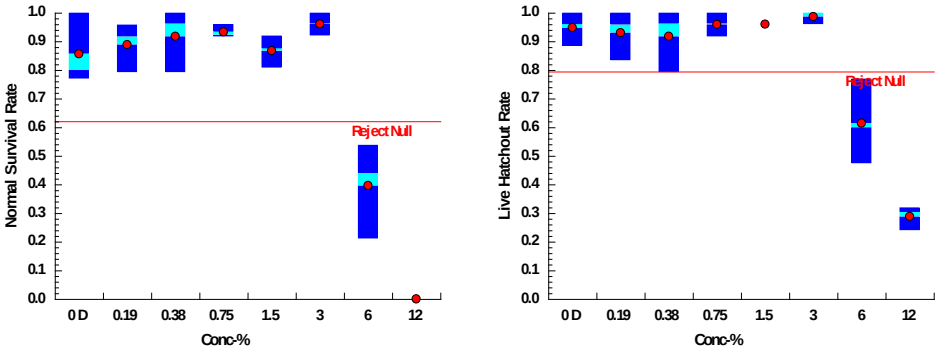
NEFemb17 – 5/16/2007 – **Aluminum Smelter Effluent**



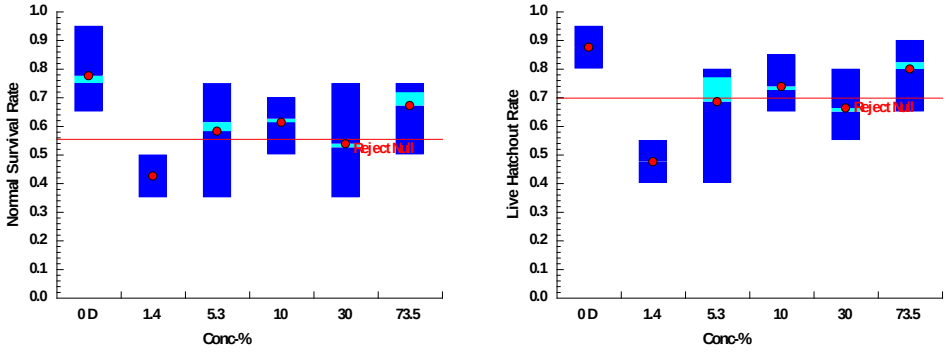
SPMCemb16 – 3/21/2005 – **POTW effluent** spiked with $CuCl_2$, KCl, and SDS



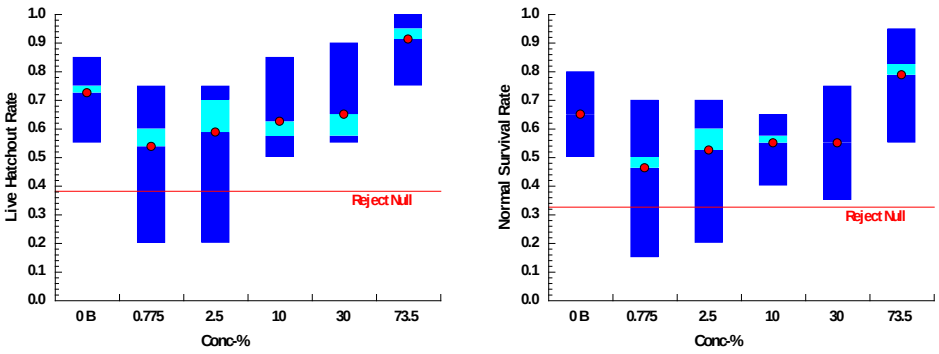
SPMCemb17 – 5/5/2005 – **POTW effluent** spiked with $CuCl_2$, KCl, and SDS



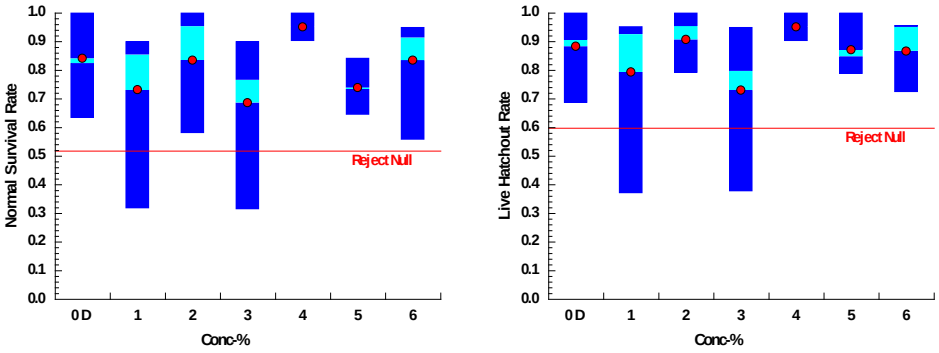
NEFemb18 – 5/4/2011 – **POTW effluent** (unmodified sample from a WWTP)



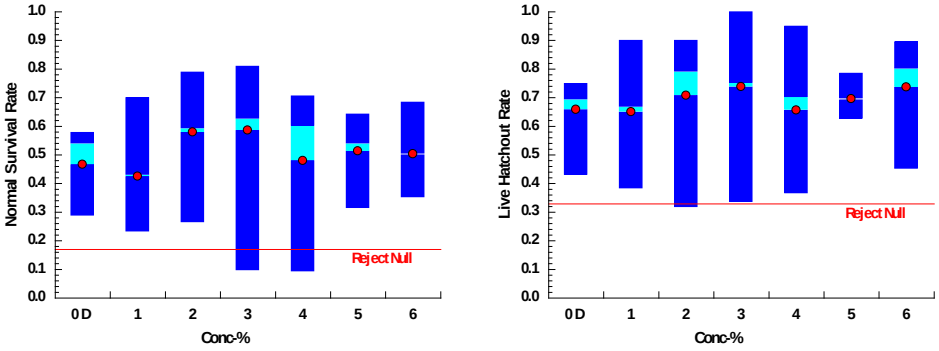
NEFemb19 – 5/4/2011 – **POTW effluent** (unmodified sample from another WWTP)



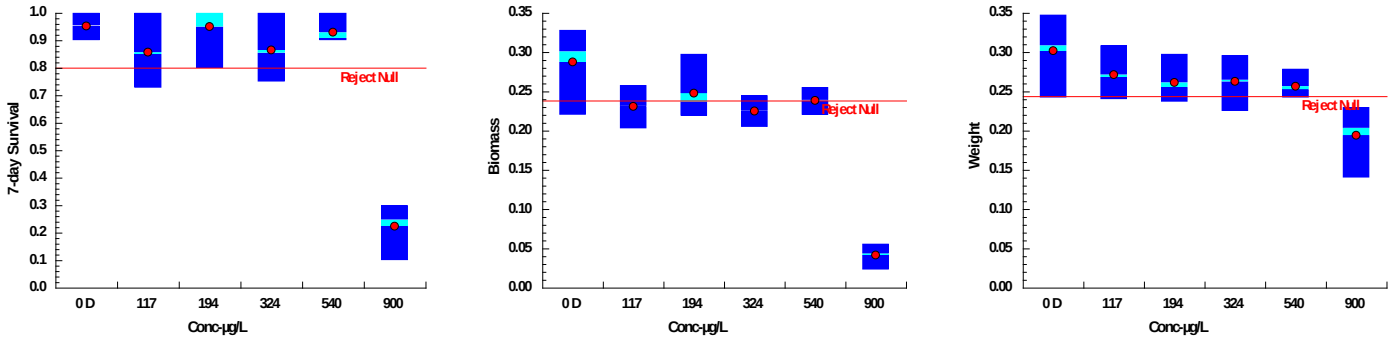
SPMCemb18 – 5/15/2007 – **Cherry Point Reach** samples from 3 stations (odd # shallow; following even # deep)



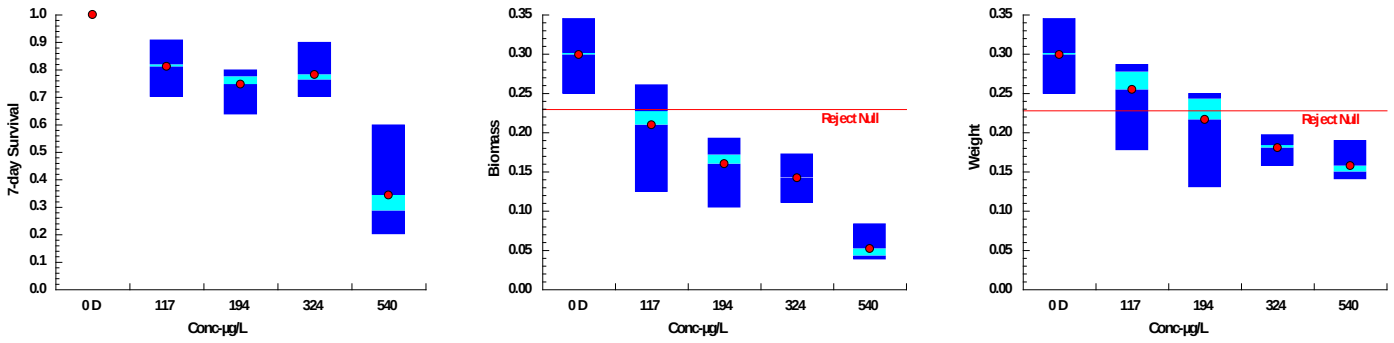
SPMCemb19 – 5/30/2007 – **Cherry Point Reach** samples from 3 stations (odd # shallow; following even # deep)



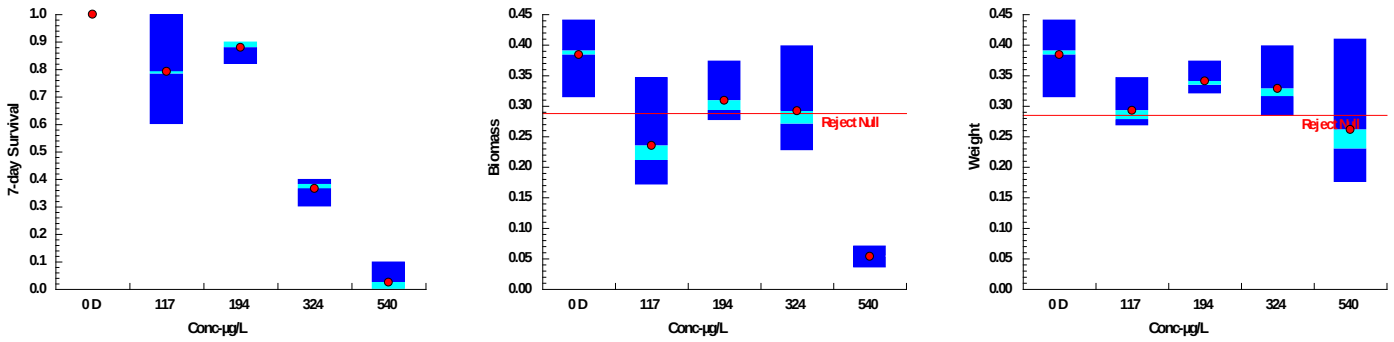
SPMClarv01 – 3/8/2010 – **$CuCl_2$**



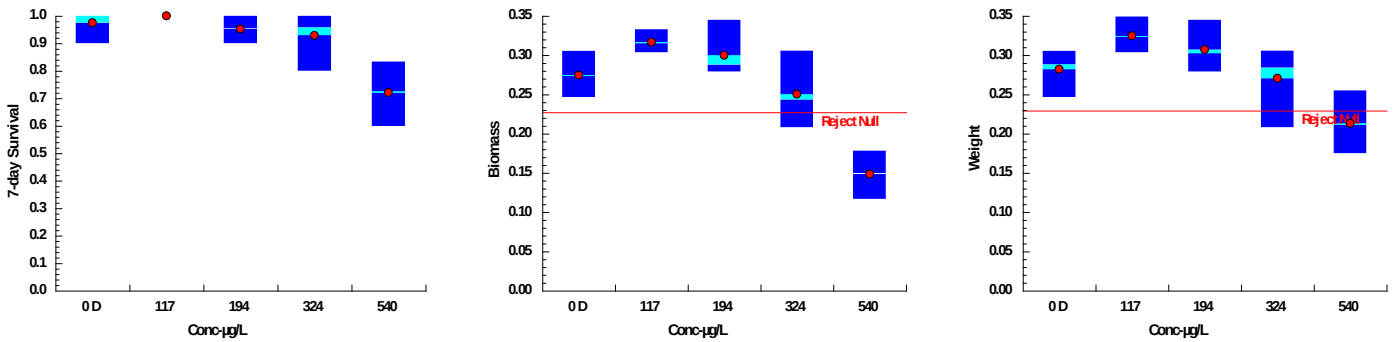
SPMClarv02 – 4/6/2010 – **$CuCl_2$**



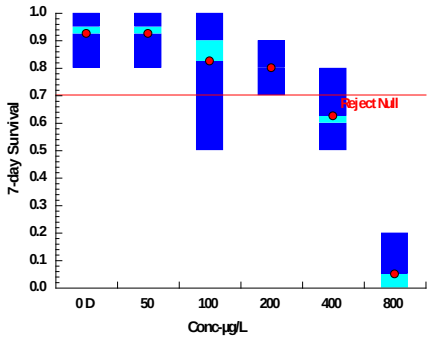
SPMClarv03 – 4/14/2010 – **$CuCl_2$**

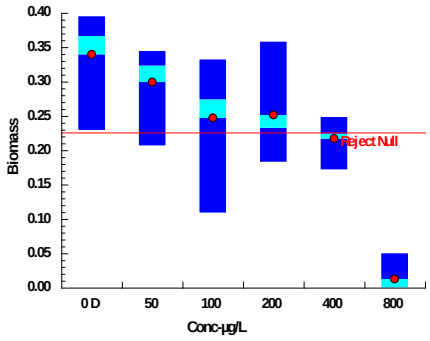


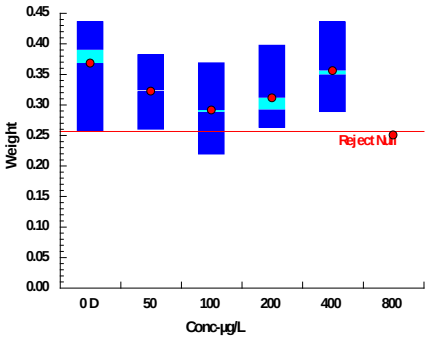
SPMClarv04 – 2/9/2011 – **$CuCl_2$**



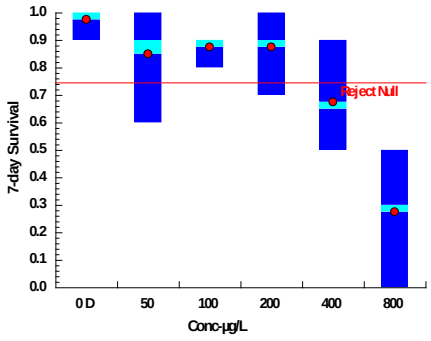
NEFlarv01 – 4/5/2010 – **$CuCl_2$**

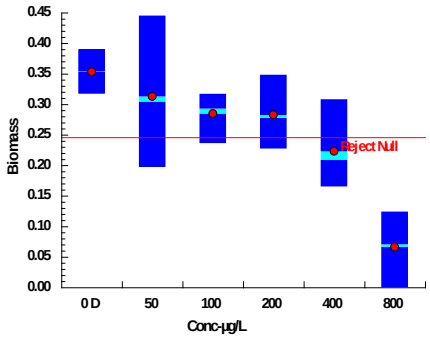


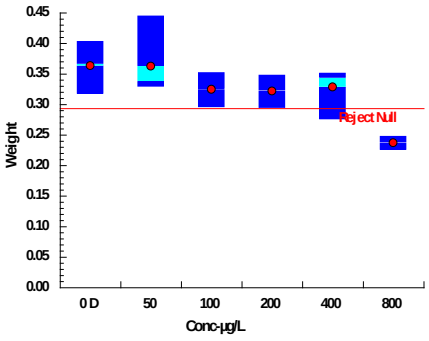




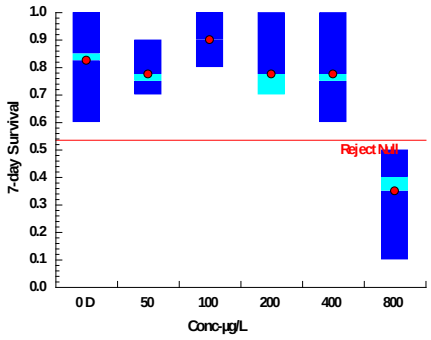
NEFlarv02 – 4/12/2010 – **$CuCl_2$**

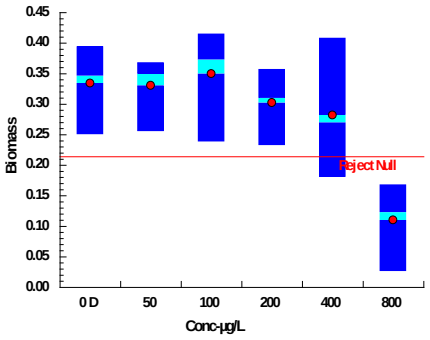


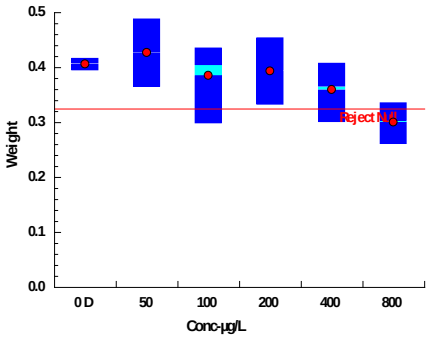




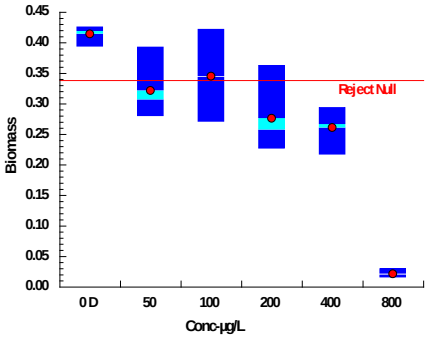
NEFlarv03 – 5/17/2010 – **$CuCl_2$**

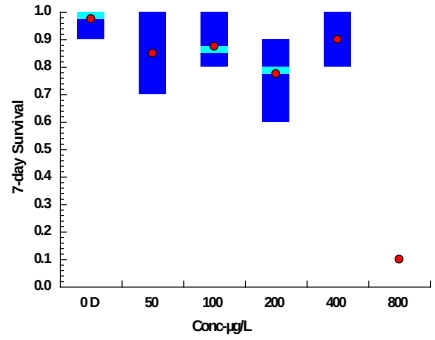


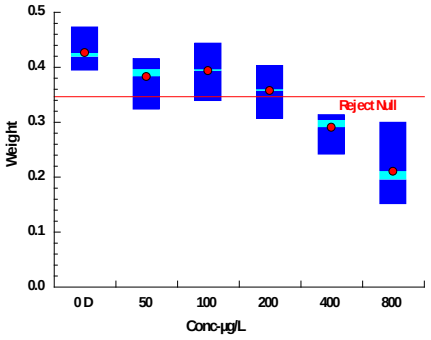




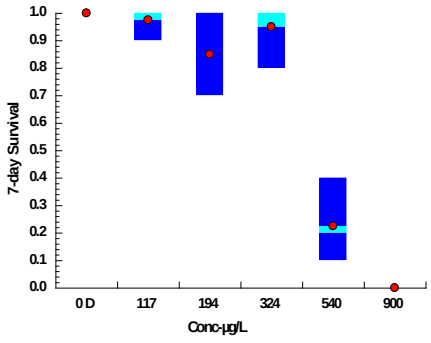
NEFlarv04 – 3/2/2011 – **$CuCl_2$**

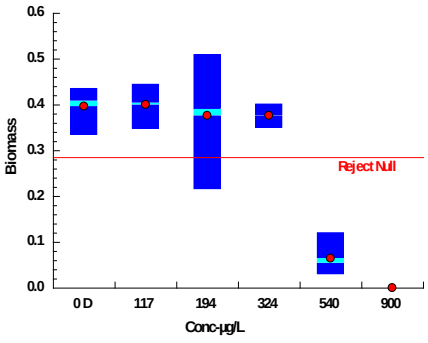


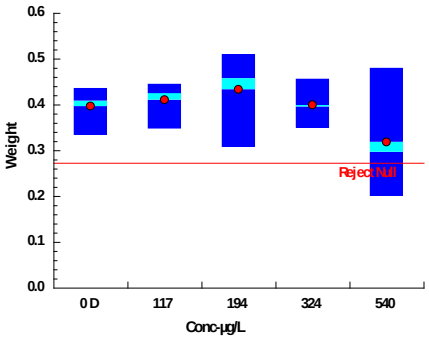




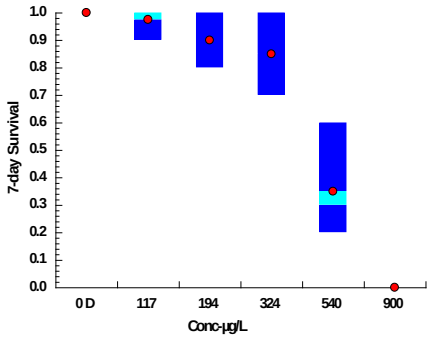
NEBlarv01 – 4/13/2010 – **$CuCl_2$**

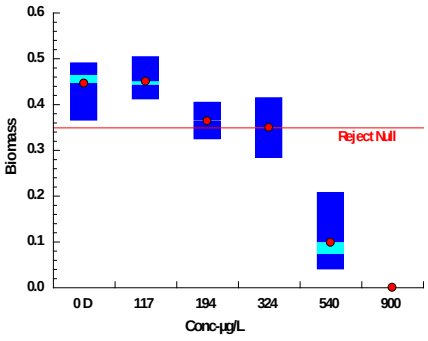


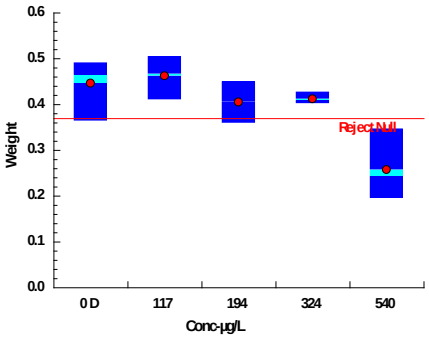




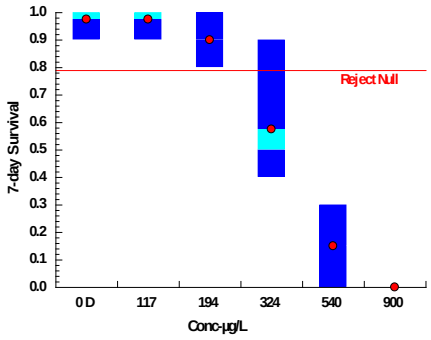
NEBlarv02 – 4/13/2010 – **$CuCl_2$**

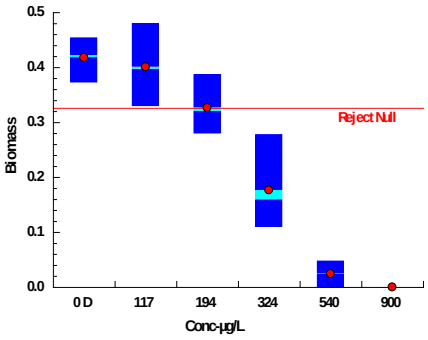


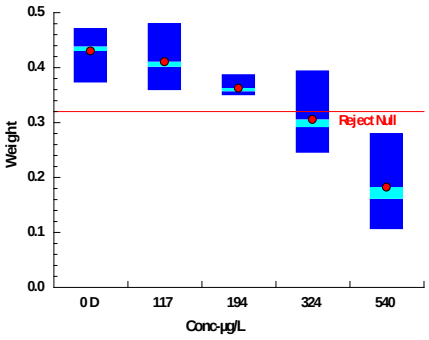




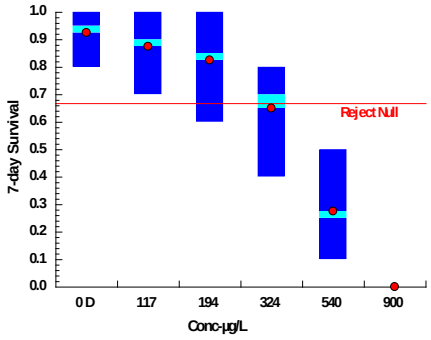
NEBlarv03 – 4/14/2010 – **$CuCl_2$**

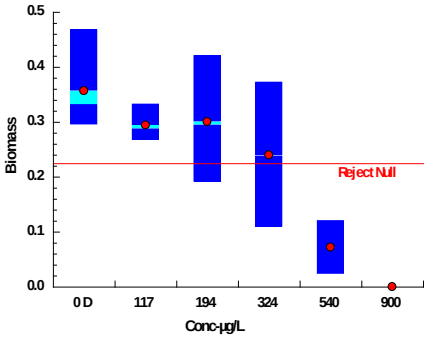


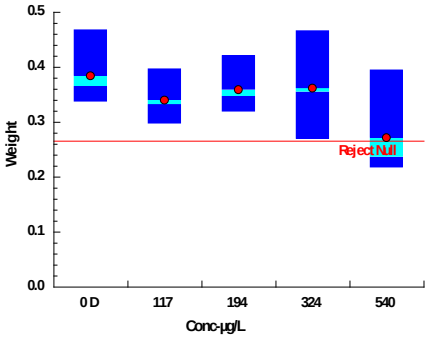




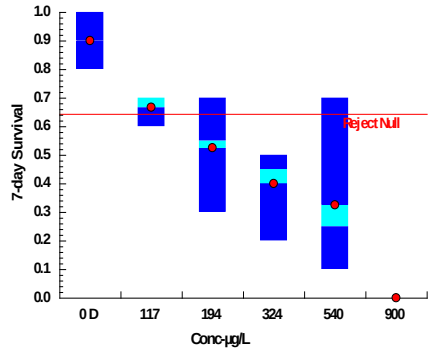
NEBlarv04 – 4/14/2010 – **$CuCl_2$**

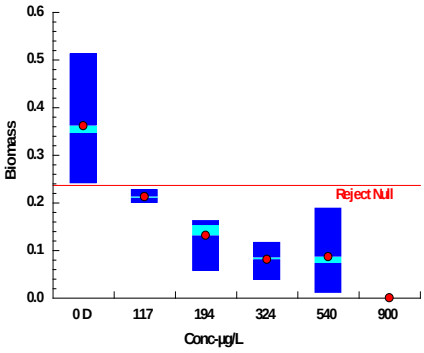


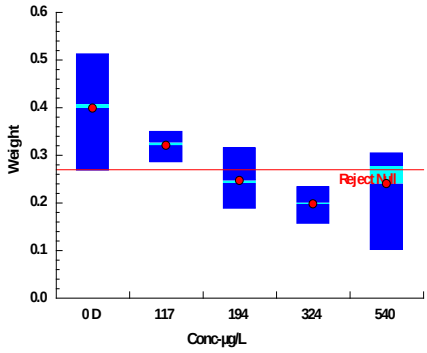




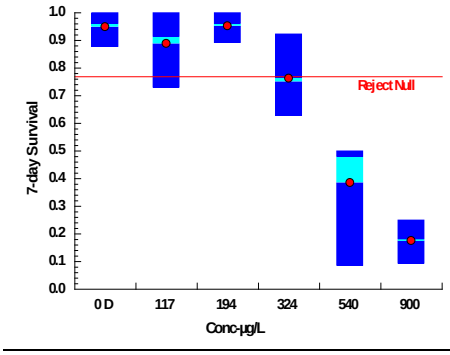
NFlarv01 – 4/12/2010 – **$CuCl_2$**

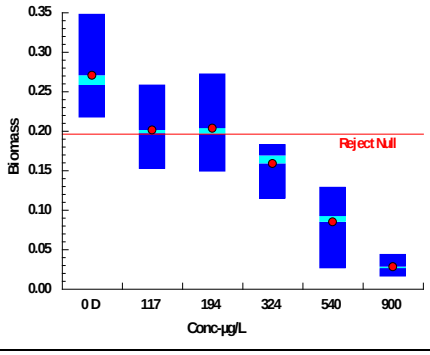


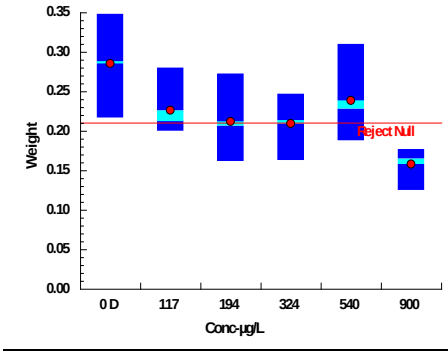




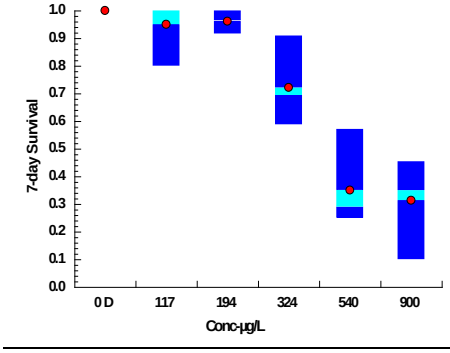
NFlarv02 – 2/11/2011 – **$CuCl_2$**

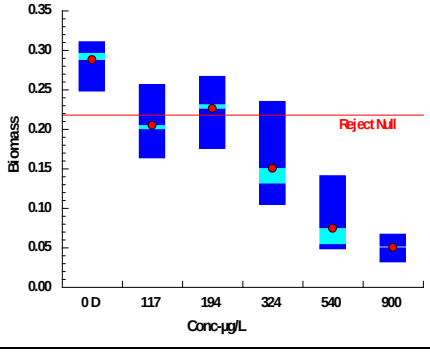


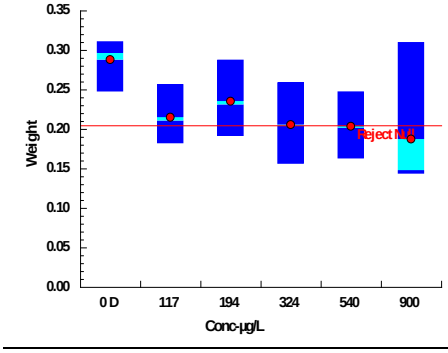




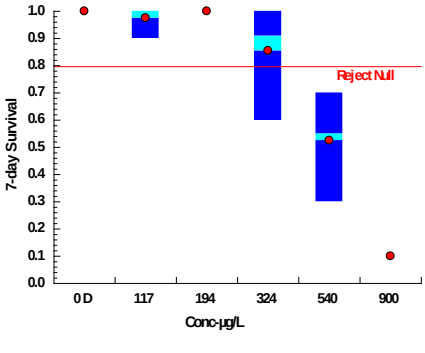
NFlarv03 – 2/11/2011 – **$CuCl_2$**

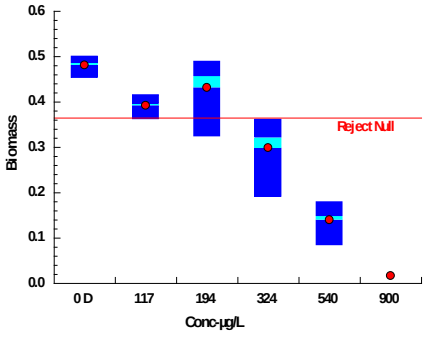


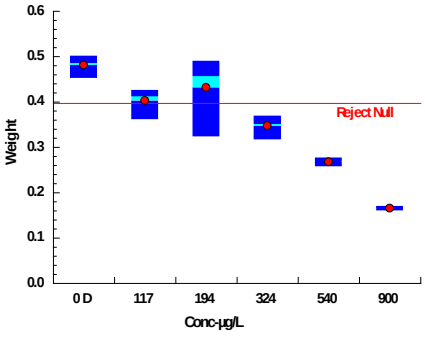




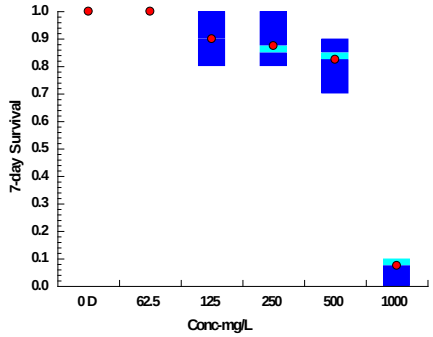
NFlarv04 – 2/15/2011 – **$CuCl_2$**

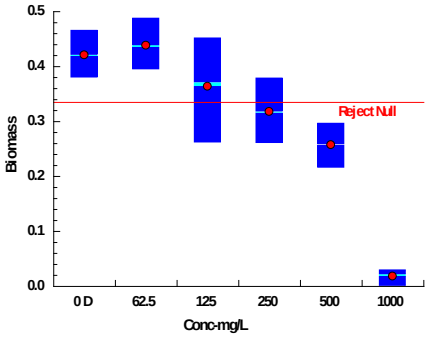


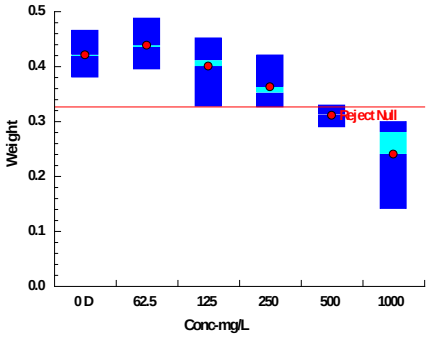




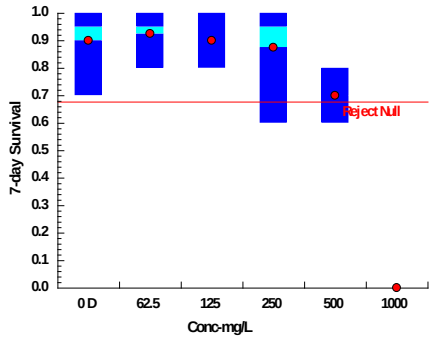
NEFlarv05 – 4/6/2010 – **KCl**

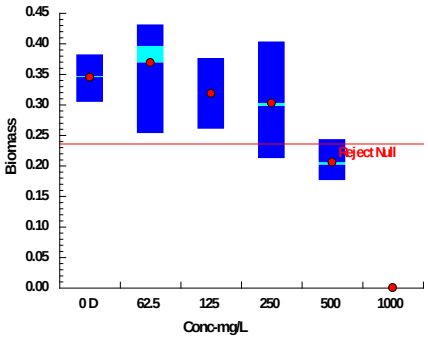


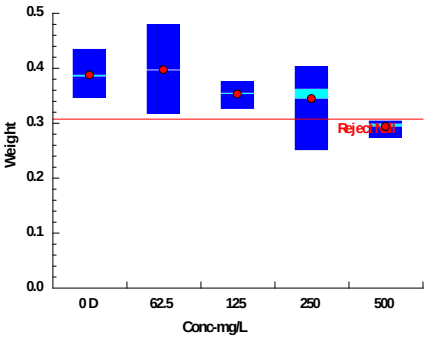




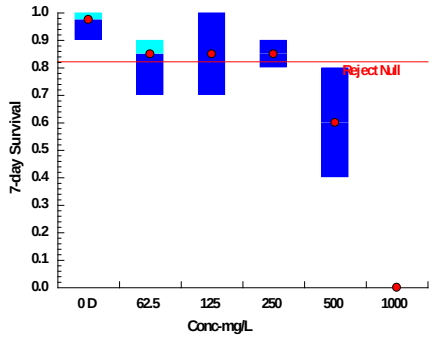
NEFlarv06 – 4/13/2010 – **KCl**

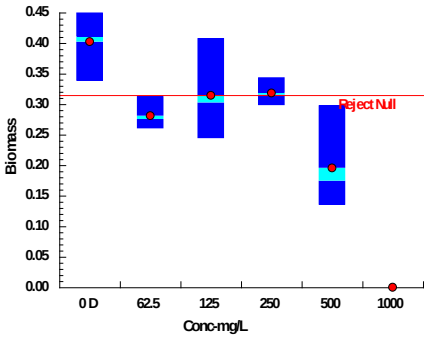


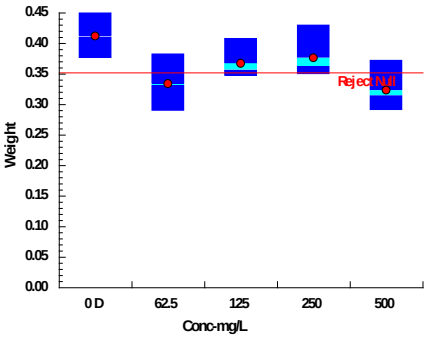




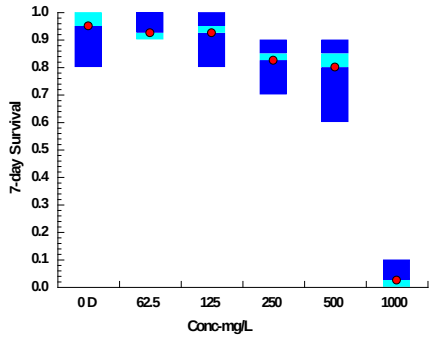
NEFlarv07 – 5/18/2010 – **KCl**

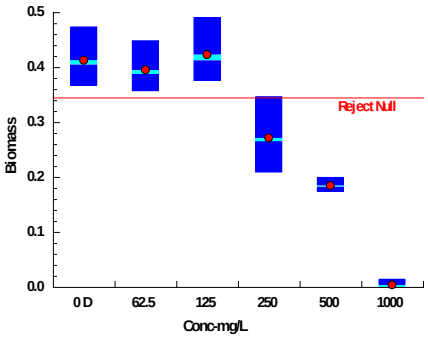


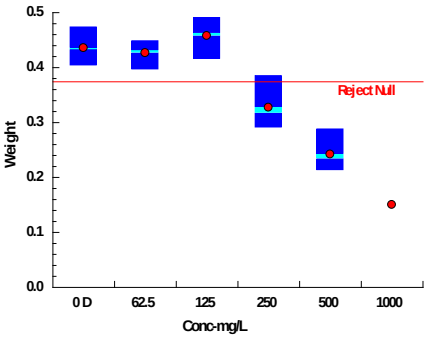




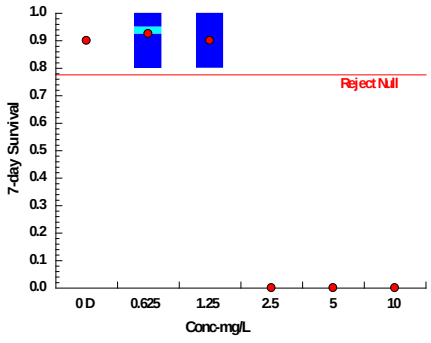
NEFlarv08 – 5/18/2010 – **KCl**

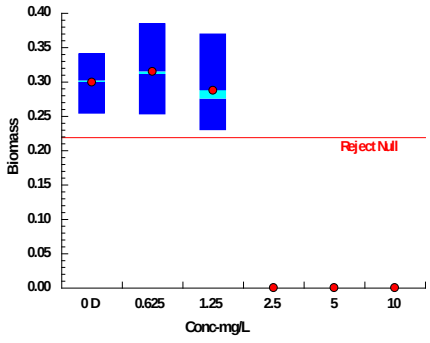


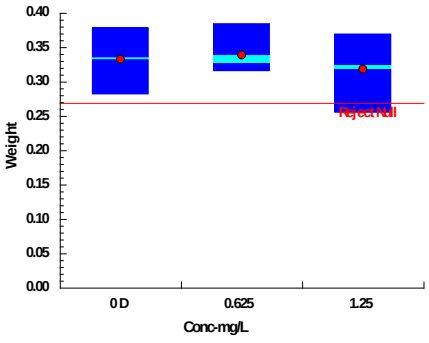




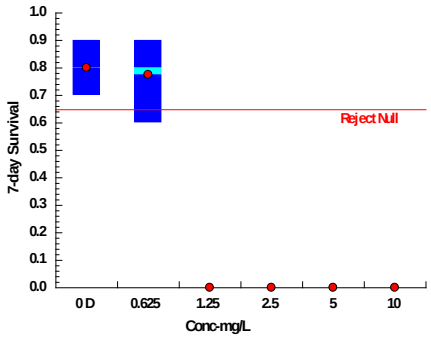
NEFlarv09 – 4/14/2010 – **SDS**

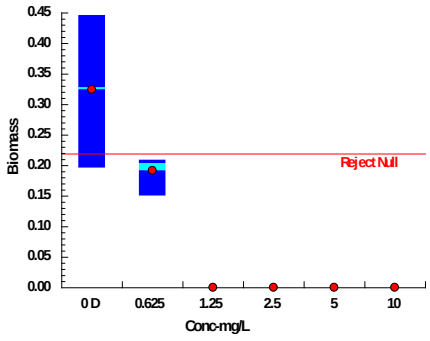


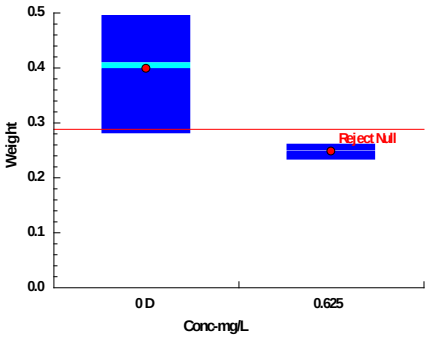




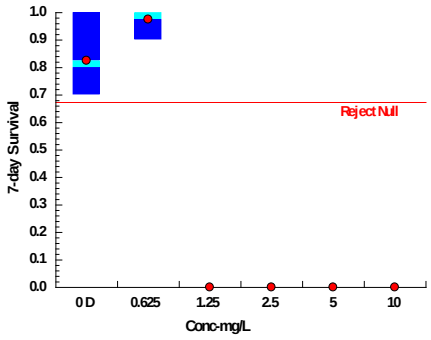
NEFlarv10 – 5/19/2010 – **SDS**

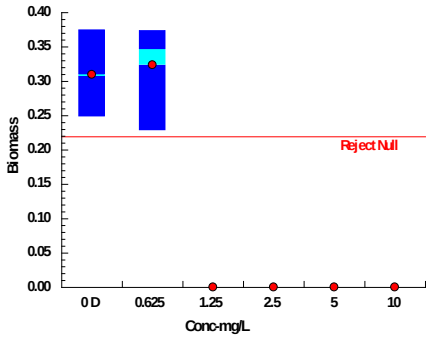


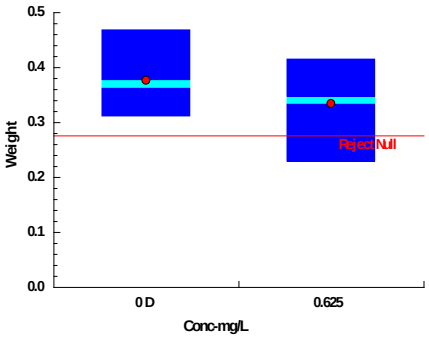




NEFlarv11 – 5/19/2010 – **SDS**







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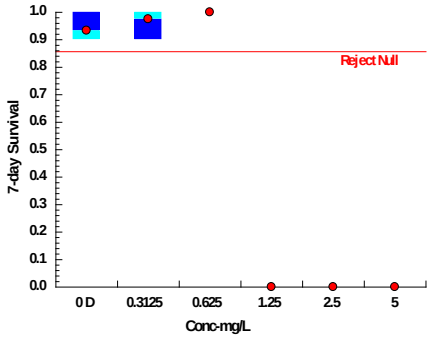

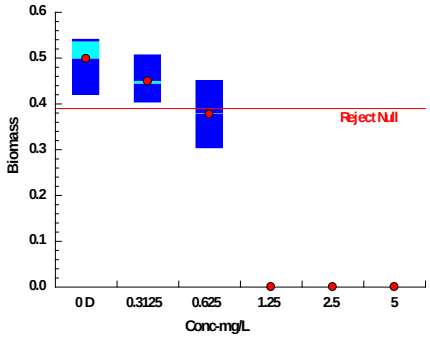

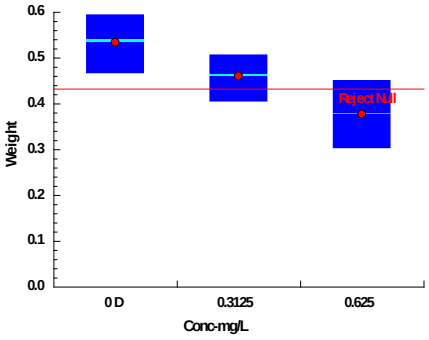

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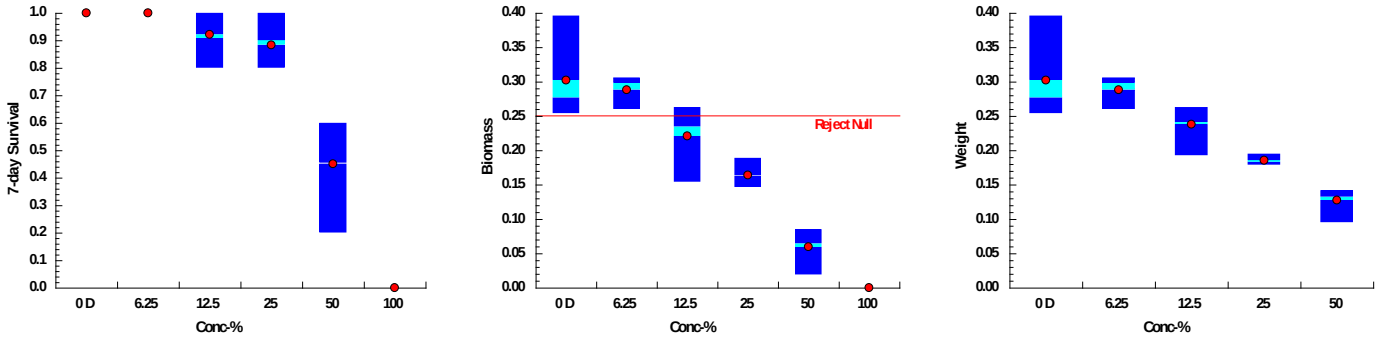

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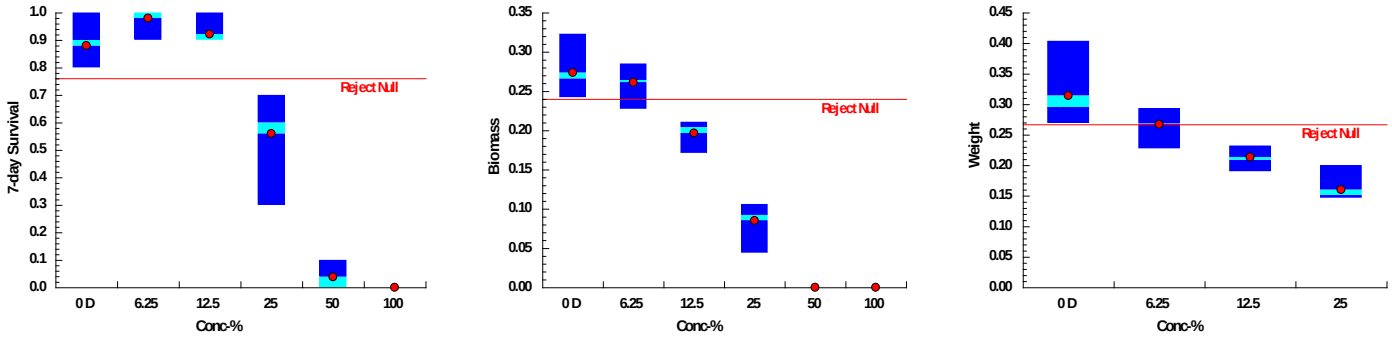

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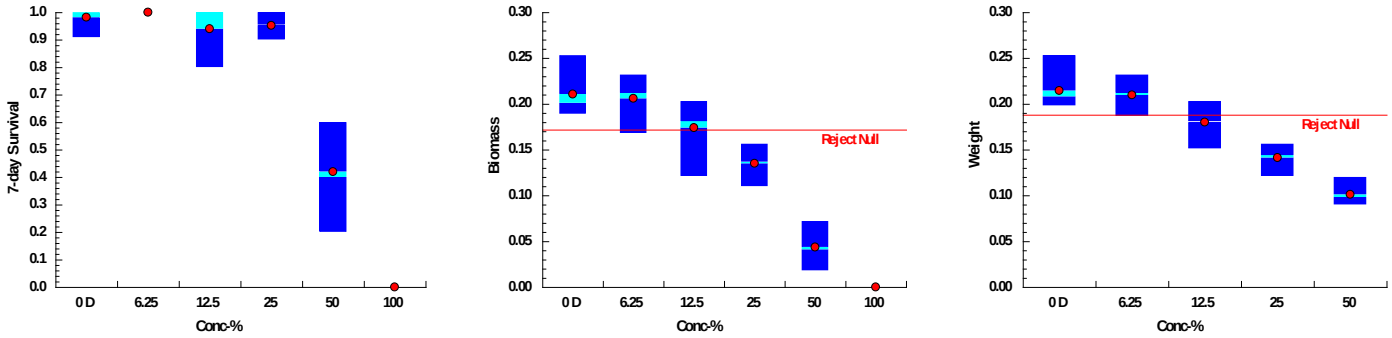
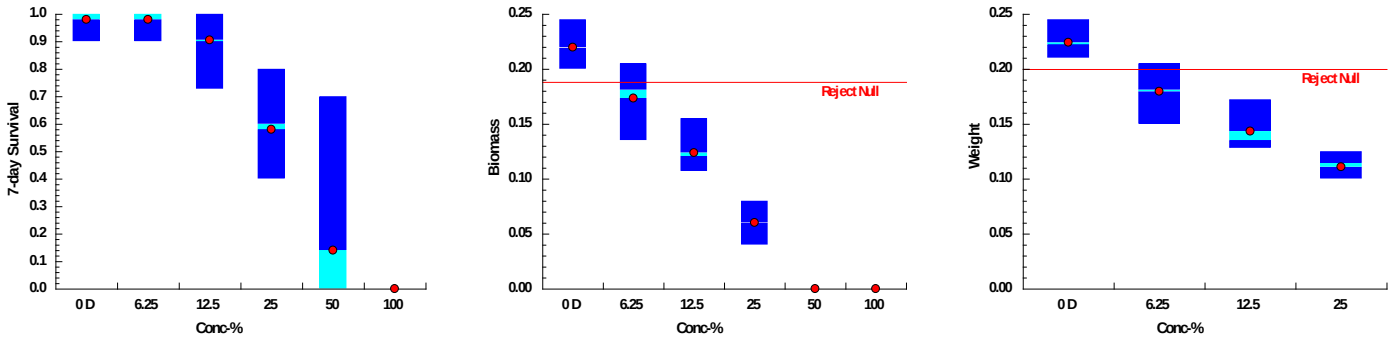
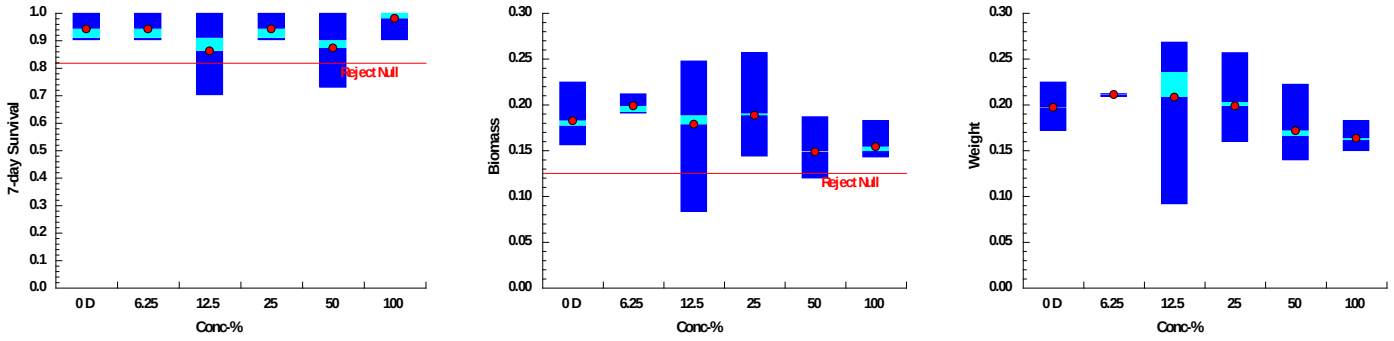


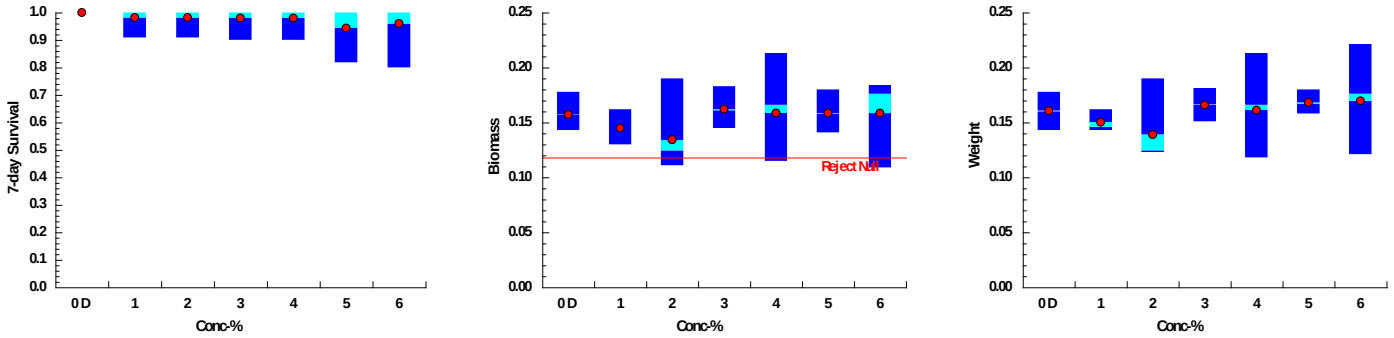
SPMClarv08 – 6/15/2007 – **Oil Refinery 4 Effluent**



SPMClarv09 – 5/16/2007 – **Aluminum Smelter Effluent**



SPMClarv10 – 5/15/2007 – **Cherry Point Reach** samples from 3 stations (odd # shallow; following even # deep)



Appendix C. Embryo Temperature Tolerance Comparisons of West Coast Stocks

Background

Herring deposit eggs in shallow water and sometimes even in the intertidal zone. Herring embryos can be exposed to heat through water, air, or sunlight. The degree of heat exposure will vary between herring stocks depending on the latitude and time of year for spawning. The Cherry Point herring spawn in late spring raising concern about the role warm temperatures might play in the decline in recruitment. However, the Cherry Point herring may have acquired tolerance for the warm temperatures common during their spawning season. It made sense to test the hypothesis that Cherry Point herring are more tolerant of heat than other regional herring by comparing the temperature tolerance of embryos from the stocks we receive for toxicity testing purposes from spawning grounds from San Francisco to Alaska.

Given the variety of toxicity test species and protocols used routinely, commercial testing labs must be able to test on any given day at multiple test temperatures. Since this was the case with the labs participating in herring toxicity test validation, we had a convenient opportunity to expose test chambers containing newly fertilized herring embryos to a series of temperatures and determine a temperature-response relationship. If enough of these temperature-response relationships could be generated to perform statistics, then statistical analysis could reveal whether embryos from different herring stocks have varying tolerance for warm temperatures related to spawning location or timing.

The temperature tolerance test method followed the same protocol (Dinnel et al, 2011) as for the herring embryo survival & development test except that replicates of four test chambers were held in separate incubators at 10°, 12°, 15°, 18°, and 20° C. The labs successfully completed all tests except:

- One of the Puget Sound tests had only 11% live hatch and 0% normal survival at 12° C and this is obvious in Figure 1 and Figure 2. The lab reported that the test chambers from this test were subjected to constant vibration from an aeration pump in the 12° C incubator.
- One Puget Sound test was conducted without 18° C so n= 1 for the mean at that temperature.
- For similar reasons, the Strait of Georgia data has n = 2 for the means at 15° and 18° C.

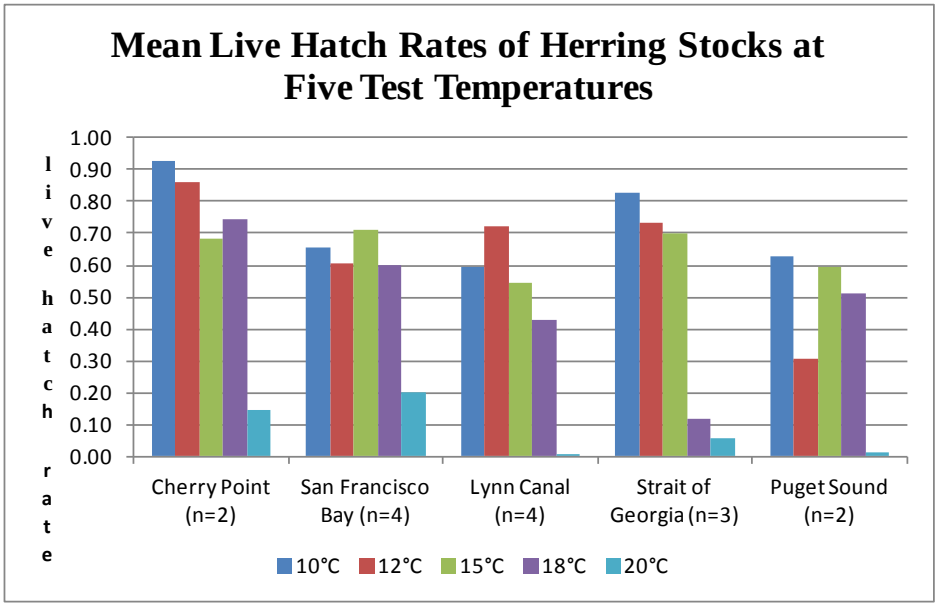


Figure 2. Live Hatch Rate Responses to Five Temperatures

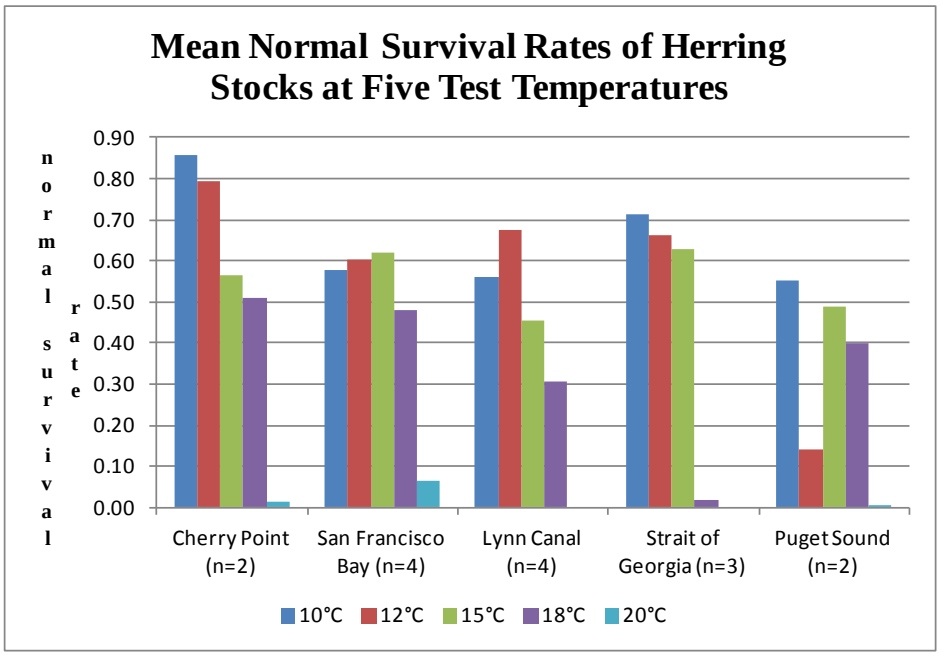


Figure 3. Normal Survival Rate Responses to Five Temperatures

Results

The threshold for embryo temperature response in Figure 2 and Figure 3 looks to be around 18° C for all stocks. Below 18° C other influences seem to be affecting response and produce no consistent trends between temperatures or stocks. However, most of the herring test results at 18° C show reduced live hatch and all show reduced normal survival. Comparisons of embryo responses at 18° and 20° C more clearly show differences in temperature tolerance between herring stocks. Figure 4 and Figure 5 illustrate the differences in response between the five herring populations at just these two temperatures.

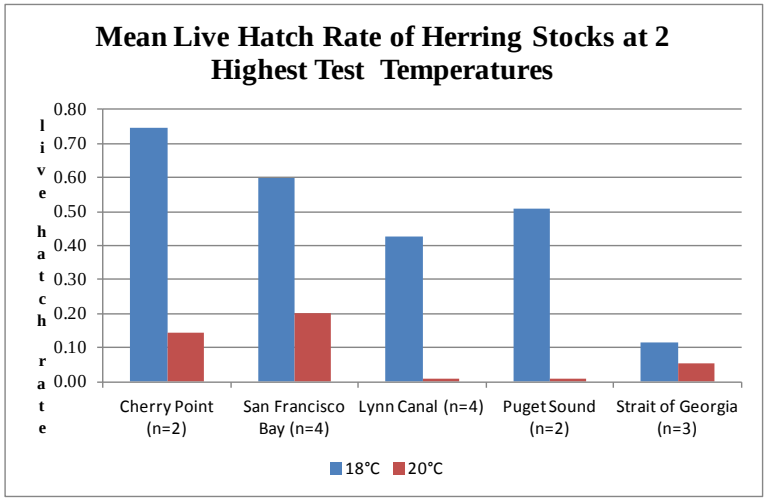


Figure 4. Live Hatch Rate Responses at Two Highest Test Temperatures

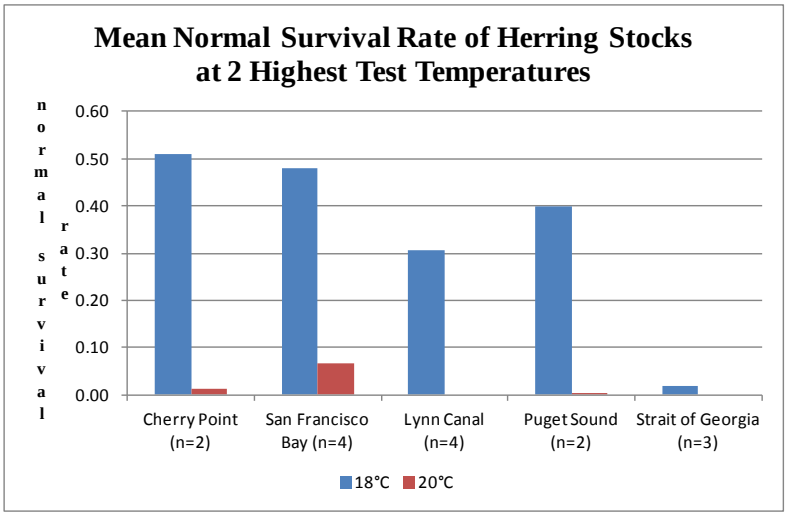


Figure 5. Normal Survival Rate Responses at Two Highest Test Temperatures

Table 9 shows the results of statistical comparisons of the temperature tolerance of Cherry Point herring embryos to those of the other four herring stocks. Because of the low number of temperature tolerance tests for Cherry Point, Strait of Georgia, and Puget Sound, statistical comparisons needed to be based upon the test organism responses in individual test chambers. There were four separate test chambers (replicates) run at each temperature in order to account for variability in test organism response. The number of data points available for analysis is the number of temperature tolerance tests times four. Table 9 shows the total number (N) of replicate responses used in each set of statistical comparisons.

SPSS 14.0 provided the statistical analyses for the temperture data. The Shapiro-Wilk test determined if datasets were normally distributed and the Levene test determined if the two datasets being compared had equal variances. The choice of mean comparison calculation was based upon $p < 0.01$ as the cutoff for rejecting the null hypothesis that the data were normally distributed or had equal variances. Because of the mix of results for the assumptions tests, Table 9 provides 2-tailed probability (p) values for the nonparametric Mann-Whitney U test and for both equal and unequal variance t-tests. Discussions below use $p < 0.05$ as the cutoff for rejecting the null hypothesis that temperature tolerance was not different.

The results in Table 9 show:

- **For live hatch at 18° C**, the Strait of Georgia ($p = 0.0000003$), Lynn Canal ($p= 0.006$), and Puget Sound ($p = 0.03$) herring were significantly different from Cherry Point. The Cherry Point and San Francisco Bay herring difference was not significant with $p = 0.18$.
- **For normal survival at 18° C**, the Strait of Georgia was significantly different ($p < 0.001$) from Cherry Point. The difference from Lynn Canal was not quite significant with $p = 0.09$. The differences between the Cherry Point and San Francisco Bay herring ($p = 0.77$) and Puget Sound herring ($p = 0.45$) were not significant.
- **For live hatch at 20° C**, Cherry Point herring were significantly different from Lynn Canal ($p < 0.001$) and Puget Sound ($p = 0.003$). The difference with the Strait of Georgia herring was not quite significant with $p = 0.07$. The difference between the Cherry Point and San Francisco Bay herring was not significant with $p = 0.47$.
- **For normal survival at 20° C**, the Cherry Point herring were significantly different from Lynn Canal ($p = 0.04$). The Cherry Point herring were not quite significantly different from San Francisco Bay ($p = 0.08$) or Strait of Georgia ($p = 0.08$) herring. The difference between the Cherry Point and Puget Sound herring was not significant with $p = 0.44$.

Table 9. Comparison of Cherry Point herring embryo temperature tolerance to other stocks

Probability (2-tailed p) Values Involved in Comparisons of Cherry Point Herring Temperature Tolerance to other Stocks

Live Hatch at 18° Celsius	Cherry Point	Lynn Canal	Puget Sound	San Francisco Bay	Strait of Georgia
N	8	16	4	16	8
mean	0.746	0.428	0.510	0.598	0.116
Shapiro-Wilk test for normality	0.958	0.114	0.519	0.236	0.033
Levene test for equal variance		0.047	0.316	0.020	0.550
Mann-Whitney U		0.017	0.027	0.244	0.001
equal variance t-test		0.006	0.029	0.177	0.0000003
unequal variance t-test		0.001	0.077	0.096	0.001
Live Hatch at 20° Celsius	Cherry Point	Lynn Canal	Puget Sound	San Francisco Bay	Strait of Georgia
N	8	16	8	16	12
mean	0.144	0.009	0.010	0.203	0.055
Shapiro-Wilk test for normality	0.107	0.000	0.000	0.017	0.003
Levene test for equal variance		0.000	0.000	0.023	0.019
Mann-Whitney U		0.000	0.003	0.733	0.072
equal variance t-test		0.0002	0.008	0.465	0.053
unequal variance t-test		0.016	0.016	0.387	0.090
Normal Survival at 18° Celsius	Cherry Point	Lynn Canal	Puget Sound	San Francisco Bay	Strait of Georgia
N	8	16	4	16	8
mean	0.511	0.306	0.400	0.480	0.018
Shapiro-Wilk test for normality	0.063	0.025	0.734	0.447	0.000
Levene test for equal variance		0.923	0.022	0.400	0.000
Mann-Whitney U		0.084	0.392	0.668	0.000
equal variance t-test		0.090	0.450	0.771	0.0009
unequal variance t-test		0.097	0.339	0.782	0.001
Normal Survival at 20° Celsius	Cherry Point	Lynn Canal	Puget Sound	San Francisco Bay	Strait of Georgia
N	8	16	8	16	12
mean	0.013	0.000	0.005	0.066	0.000
Shapiro-Wilk test for normality	0.000	NC	0.000	0.004	NC
Levene test for equal variance		0.000	0.093	0.014	0.000
Mann-Whitney U		0.041	0.440	0.082	0.075
equal variance t-test		0.038	0.447	0.083	0.074
unequal variance t-test		0.170	0.450	0.024	0.170

Some of the p values from comparison of the means from Cherry Point and San Francisco Bay herring are quite large. The comparison of normal survival at 18° C provided p = 0.77. To refine the impression that a large p value represents similarity between these stocks, a one-sample t-test was done of Cherry Point normal survival data versus the mean normal survival rate (0.48) at 18° C for San Francisco Bay. The comparison yielded p = 0.75 allowing acceptance of the null hypothesis that Cherry Point normal survival at 18° C equals 0.480 (the mean normal survival rate for San Francisco Bay). The null hypothesis that the Cherry Point herring response equaled the mean for another stock was also accepted for comparisons to San Francisco Bay live hatch at 20° C (p = 0.21) and Puget Sound normal survival at 18° C (p = 0.28).

San Francisco Bay and Lynn Canal both have four temperature tolerance test results and provide a good opportunity for statistical comparisons of point estimates derived from the responses at all temperatures for each test. Test organism responses at a series of temperatures are the same mathematically as test organism responses at a series of chemical concentrations. Median effect levels such as the LC50 are the standard metric in toxicology for comparing the toxicity of different chemicals or the sensitivity of different species. CETIS was used to calculate median effect level point estimates using herring embryo responses at all five test temperatures. Spearman-Kärber provided point estimates for the median live hatch effect level and linear regression (probit model) provided point estimates for the median effect level for normal survival. These point estimates allowed additional statistical comparisons of the difference in response between stocks using data from all of the test temperatures.

Because they are the southernmost and northernmost herring stocks in the study, the data from the San Francisco Bay and Lynn Canal herring stocks also provide an opportunity to test the hypothesis that herring embryos from southern stocks have greater tolerance for warm temperatures. Table 10 presents 1-tailed probability (p) values to test this hypothesis. Table 10 also contains comparison results for replicate responses at 18° and 20° C similar to Table 9 of comparisons of Cherry Point to the other stocks.

The Shapiro-Wilk test determined if datasets were normally distributed and the Levene test determined if the two datasets being compared had equal variances. The choice of mean comparison calculation was based upon $p < 0.01$ as the cutoff for rejecting the null hypothesis that the data were normally distributed or had equal variances. Because of the mix of results for these assumptions tests, Table 10 provides 1-tailed probability (p) values for the nonparametric Mann-Whitney U test and for both the equal and unequal variance t-tests. The discussion below uses $p < 0.05$ as the cutoff for rejecting the null hypothesis that temperature tolerance was not different between San Francisco Bay and Lynn Canal.

Table 10 uses the "EL50" for median effect levels for herring temperature responses because the "ET50" is standard toxicological nomenclature for responses related to time. The results in Table 10 show:

- Comparisons of live hatch ($p = 0.048$) and normal survival ($p = 0.03$) show San Francisco Bay herring to be significantly more tolerant of embryo exposure to 18° C than Lynn Canal herring.
- Comparisons of live hatch ($p = 0.0005$) and normal survival ($p = 0.0005$) show San Francisco Bay herring to be significantly more tolerant of embryo exposure to 20° C than Lynn Canal.
- The median effect temperature for live hatch from San Francisco Bay herring was significantly higher ($p = 0.014$) than the median effect temperature for Lynn Canal. The difference between median effect temperatures for normal survival was almost significant with $p = 0.0505$.

Table 10. Comparison of San Francisco Bay to Lynn Canal herring embryo temperature tolerance

Probability (p) Values Involved in Comparisons of San Francisco Bay Herring Temperature Tolerance to Lynn Canal

Assumptions tests are 2-tailed and comparison of means tests are 1-tailed.	Live Hatch			Normal Survival		
	EL50	18° C	20° C	EL50	18° C	20° C
N	4	16	16	4	16	16
San Francisco Bay mean	19.5° C	0.598	0.203	18.6° C	0.480	0.066
Lynn Canal mean	17.2° C	0.428	0.009	16.8° C	0.306	0.000
Shapiro-Wilk test - San Francisco Bay	0.360	0.236	0.017	0.060	0.447	0.004
Shapiro-Wilk test - Lynn Canal	0.107	0.114	0.000	0.171	0.025	NC
Levene test for equal variance	0.007	0.783	0.000	0.000	0.388	0.000
Mann-Whitney U	0.0105	0.068	0.0005	0.0105	0.027	0.0005
equal variance t-test	0.0085	0.048	0.0005	0.032	0.0305	0.0015
unequal variance t-test	0.014	0.048	0.001	0.0505	0.0305	0.0025

Figure 6 (live hatch) and Figure 7 (normal survival) illustrate the comparisons of median effect temperature estimates.

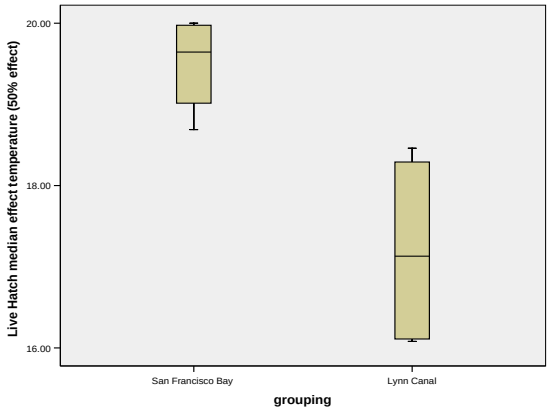


Figure 6. Comparison of Live Hatch Median Effect Levels for San Francisco Bay and Lynn Canal

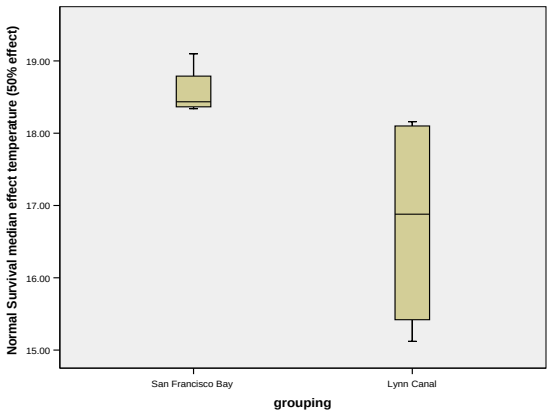


Figure 7. Comparison of Normal Survival Median Effect Levels for San Francisco Bay and Lynn Canal

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Conclusions

The comparison of San Francisco Bay herring temperature tolerance to Lynn Canal demonstrates the utility of the approach when at least four test results are available. As hypothesized, the San Francisco Bay herring embryos were significantly more tolerant of higher water temperature than Lynn Canal based upon comparisons of live hatch and normal survival measurements at two temperatures. Comparisons of the median effect levels for live hatch showed a significant difference that accounted for responses at all five test temperatures. The difference in normal survival median effect level was close to significant.

Statistical comparisons showed Cherry Point herring to have significant differences from Lynn Canal and Strait of Georgia herring in embryo live hatch and normal survival response at 18° and 20° C. Cherry Point herring were significantly different from Puget Sound for live hatch, but demonstrated some similarity for normal survival. The San Francisco Bay and Cherry Point herring embryo results did not demonstrate statistically significant differences in any of the comparisons. Statistics found the hypotheses acceptable that Cherry Point response for normal survival at 18° C and live hatch at 20 C equaled the mean responses for San Francisco Bay. This relationship is as predicted given the late spring spawning of the Cherry Point herring and distance south for the San Francisco Bay spawning grounds.

More temperature tolerance testing is needed. All of the stocks involved in this study should be brought up to a minimum of four tests a piece. In addition, it is important to determine whether the differences seen in temperature tolerance are due to genetics or to environmental conditioning. The information may prove to be a key consideration for resource management in a changing climate.