

May 21, 2020

Mr. Christopher Bittner
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Subject: Results of Acute Brine Shrimp Experiment #15

Mr. Bittner/ Dr. Belovsky:

Below is a summary of the acute brine shrimp experiment initiated on May 14, 2020. The purpose of this experiment was to investigate an appropriate range for a short term chronic *Artemia franciscana* test with zinc as the toxicant.

Along with a control, five different nominal zinc concentrations (prepared with ZnSO₄) were tested:

- 19, 37.5, 75, 150, and 300 mg/L

The results of these studies will help determine the experimental design of the definitive short-term chronic toxicity tests. The test volume was consistent at 50 ml.

Species: *Artemia franciscana*

Test type:

- Test duration: 4 days
- Test type: static-renewal (solutions and food renewed daily)
- Algae: *Dunaliella viridis*
- Food concentration: 72.5 µg/L Ch1a and 0.3 ml YTC¹
- Temperature: 20°C
- Test volume(s): 50 ml
- Replicates: 4
- Organisms/Rep: 10
- Test media: 120 ppt rGSL media (per Notre Dame recipe)

Pretest conditions: <24-h old *A. franciscana* were hatched out in ~29 ppt artificial seawater (Crystal Sea Marine Mix) and ~200 organisms were placed in 120 ppt rGSL water and fed *Dunaliella viridis* at a density of 72.5 µg/L Ch1a and 0.3 ml YTC. Solutions were gently aerated.

¹ yeast-trout chow-cerophyl mixture used as a typical food for water fleas in whole effluent toxicity testing (USEPA 2002)

Characterization of Recon Water

Sample No.	pH	Hard. (mg/L) ^a	Alk. (mg/L) ^a	Spec. Cond. (μS/cm)	TRC (mg/L) ^b	NH ₃ -N (mg/L)	Salinity (ppt)
RW#13930	7.9	NM	NM	138,100	NM	NM	120

^aAs CaCO₃

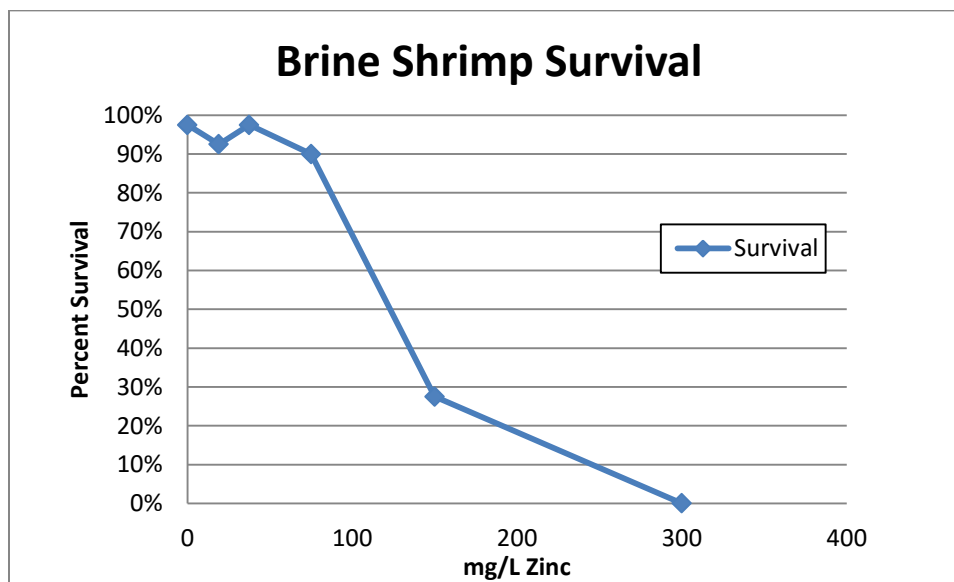
^bTotal residual chlorine

Test activities:

- Biological observations (primarily survival) taken daily.
- Chemistries taken on daily (i.e., pH, dissolved oxygen, and temperature).
- Conductivity was measured at test termination or when there was 0% survival in that treatment.
- Zinc was added to 120 rGSL media containing food and allowed to equilibrate for 3 hours prior to use in the toxicity tests.

Results:

The survival of the brine shrimp in the zinc treatments is illustrated in the following figure:



Test Endpoints

Test Concentration (mg/L Zinc) (nominal)	Percent Survival of <i>Artemia franciscana</i>			
	24 hours	48 hours	72 hours	96 hours
0 (rGSL)	97.5	97.5	97.5	97.5
19	100	92.5	92.5	92.5
37.5	97.5	97.5	97.5	97.5
75	95	92.5	90	90
150	65	40	37.5	27.5
300	20	17.5	5	0
Control Performance	Acceptable			

Data Analysis and Test Endpoints

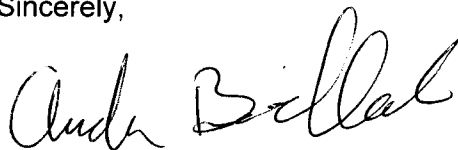
Biological Endpoint	Statistical Endpoint	Value (mg/L Zinc) (nominal)
Survival	96-hour LC ₅₀	125 (C.L. 160 -141)

Summary and findings:

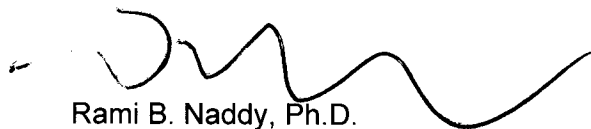
- Organism survival was ≥ 90% for the controls.
- Zinc toxicity was clearly demonstrated at these testing concentrations.
- Samples were collected for zinc analysis.
- Zinc concentrations for a short term chronic test will be similar to those used here.

We greatly appreciate the opportunity to complete this study for you. Please do not hesitate to call if you have any questions or concerns.

Sincerely,



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17001-474-069

Attachment

cc: David Pillard, TRE

TOXICITY DATA PACKAGE COVER SHEET

QA: DAF 5/19/20

Test Type: Chronic Project Number: 17001-474-069
Test Substance: Znc (ZnSO4) Species: Artemia franciscana
Dilution Water: rGSL Organism Lot or Batch Number: 051220
Concurrent Control Water: NA Age: 48HR (48 hr) Supplier: TRE
Date and Time Test Began: 5/14/20 @ 1505 Date and Time Test Ended: 5/18/20 @ 1450
Protocol Number: NA Investigator(s): cp/m JS/EN

Background Information

Type of Test: Static-Renewal (48 h) pH control?: Yes No
If yes, give % CO₂: NA
Test Temperature: 20 ± 1 °C Env. Chmbr/Bath #: 25 Test Chmbrs: 147-ml cups
Photoperiod: 16 h light : 8 h dark Light intensity: 50-100 ft-c.
Test Solution Vol.: 50 ml Replicates per Treatment: 4
Length of Test: 96 hr Organisms per Replicate: 10
Type of Food and Quantity per Chamber: 72.5 ug/L Chla/ 0.3 ml YT Feeding Frequency: Initiation and Renewals

Test Substance Characterization Parameters and Frequency:

Hardness: Test Initiation Alkalinity: Test Initiation NH₃: Test Initiation TRC: Test Initiation
pH: Daily Conductivity: Daily

Test Concentrations (Volume:Volume): rGSL, 19, 37.5, 75, 150, and 300 mg/L as Zn

Agency Summary Sheet(s)?: None

Reference Toxicant Data: Test Dates: _____ to _____ IC₂₅: _____
Hist. 95% Control Limits: _____ to _____ Method for Determining Ref. Tox. Value: Linear Interpolation

Special Procedures and Considerations:
Organisms hatched 2 days prior to initiation and held in rGSL with 72.5 ug/L Chla/ 0.3 ml YTC

Appropriate correction factors have been applied to all temperatures recorded in this data package
Study Director Initials: AB Date: 5/14/20

TEST SUBSTANCE USAGE LOG

Project Number: 17001-474-069

QA: ~~DP~~ 5/19/20

	Sample 1	Sample 2	Sample 3	Sample 4
Test Substance Number	C99-093			
Test Substance Collection Date and Time	From: 5/15/20 @	From: @	From: @	From: @
	To: @	To: @	To: @	To: @
Sample Type (Grab or Comp)	MA			
Date Test Substance Received				
Dilution Water Number RW# or TRE#, circle one	13930			
Concurrent Control Water RW#	MA			
Date(s) Used	5/14/20			
	5/16/20			

Preparation of Test Solutions

Test Substance	Test Substance Volume (ml)	Dilution Water Volume (ml)	Total Volume (ml)	Test Substance Volume (ml)	Dilution Water Volume (ml)	Total Volume (ml)	Test Substance Volume (ml)	Dilution Water Volume (ml)	Total Volume (ml)
Conc. ^① mg/L Zn (% Effluent)									
0	0	250	250						
19	16	234	250						
37.5	31	219	250						
75	63	188	250						
150	125	125	250						
300	250	0	250						
	485	1015	1500						
Initials / Date	AB 5/14/20 Mixed BS								
Initials / Date	CP 5/16/20 u a								
Initials / Date									
Initials / Date									
Initials / Date									
Initials / Date									
Initials / Date									
Initials / Date									

① new 5/19/20 E

Artemia franciscana
CHRONIC BIOLOGICAL DATA

QA : Dep 5/19/20

Project Number: 17001-474-069

mg/L	Test Replicate	Number of Surviving Organisms								% survival	Remarks
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7		
0	A	10	9	9	9	9	/	/	/	97.5	
	B	10	10	10	10	10	/	/	/		
	C	10	10	10	10	10	/	/	/		
	D	10	10	10	10	10	/	/	/		
19	A	10	10	10	10	10	/	/	/	92.5	
	B	10	10	10	10	10	/	/	/		
	C	10	10	9	9	9	/	/	/		
	D	10	10	8	8	8	/	/	/		
37.5	A	10	10	10	10	10	/	/	/	97.5	
	B	10	9	9	9	9	/	/	/		
	C	10	10	10	10	10	/	/	/		
	D	10	10	10	10	10	/	/	/		
75	A	10	10	10	9	9	/	/	/	90	
	B	10	9	9	9	9	/	/	/		
	C	10	10	10	10	10	/	/	/		
	D	10	9	8	8	8	/	/	/		
150	A	10	5	4	3	2	/	/	/	27.5	
	B	10	6	2	2	2	/	/	/		
	C	10	7	4	4	3	/	/	/		
	D	10	8	6	6*	4	/	/	/		* 2 weak orgs
300	A	10	3	3	2*	0	/	/	/	* weak orgs 0	
	B	10	2	1	0	-	/	/	/		
	C	10	2	2	0	-	/	/	/		
	D	10	1	1	0	-	/	/	/		
	A						/	/	/		
	B						/	/	/		
	C						/	/	/		
	D						/	/	/		
Date:		5/14/20	5/15/20	5/16/20	5/17/20	5/18/20					
Time:		1505	1130	1555	1125	1450					
Initials:		CP/m	ES/CP	CP	CP	EN					

CHRONIC CHEMICAL DATA (INITIAL)

QA: DEP 5/18/20

Project Number:	17001-474-069
Test Species: <i>Artemia franciscana</i>	

%	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Meter #	Remarks
Conc.: 0									All Conc.	
pH	7.9	/	8.1	/	/	/	/	/	FM27	
D.O. (mg/L)	5.2	/	5.7	/	/	/	/	/	17	
Temp. (°C)	20	/	20	/	/	/	/	/	IR1	
Cond. (µS/cm)	138,100	/	137,500	/	/	/	/	/	15	
Hard. (mg/L)	/	/	/	/	/	/	/	/		
Alk. (mg/L)	/	/	/	/	/	/	/	/		
TRC (mg/L)	/	/	/	/	/	/	/	/		
NH ₃ (mg/L)	/	/	/	/	/	/	/	/		
Conc.: 19										
pH	7.8	/	7.9	/	/	/	/	/		
D.O. (mg/L)	5.1	/	5.6	/	/	/	/	/		
Temp. (°C)	20	/	20	/	/	/	/	/		
Cond. (µS/cm)	137,900	/	137,100	/	/	/	/	/		
Hard. (mg/L)	/	/	/	/	/	/	/	/		
Alk. (mg/L)	/	/	/	/	/	/	/	/		
TRC (mg/L)	/	/	/	/	/	/	/	/		
NH ₃ (mg/L)	/	/	/	/	/	/	/	/		
Conc.: 37.5										
pH	7.8	/	7.8	/	/	/	/	/		
D.O. (mg/L)	5.2	/	5.5	/	/	/	/	/		
Temp. (°C)	20	/	20	/	/	/	/	/		
Cond. (µS/cm)	137,700	/	137,000	/	/	/	/	/		
Hard. (mg/L)	/	/	/	/	/	/	/	/		
Alk. (mg/L)	/	/	/	/	/	/	/	/		
TRC (mg/L)	/	/	/	/	/	/	/	/		
NH ₃ (mg/L)	/	/	/	/	/	/	/	/		
Conc.: 75										
pH	7.4	/	7.5	/	/	/	/	/		
D.O. (mg/L)	5.1	/	5.5	/	/	/	/	/		
Temp. (°C)	20	/	20	/	/	/	/	/		
Cond. (µS/cm)	137,100	/	137,200	/	/	/	/	/		
Hard. (mg/L)	/	/	/	/	/	/	/	/		
Alk. (mg/L)	/	/	/	/	/	/	/	/		
TRC (mg/L)	/	/	/	/	/	/	/	/		
NH ₃ (mg/L)	/	/	/	/	/	/	/	/		
Date:	5/14/20		5/15/20							
Time:	1455		1545							
Initials:	CP		CP							

Note: Hardness, alkalinity, TRC, and NH₃ data appearing on this page have been transcribed from the wet chemistry log QA Form No. 084.

*Dilution/control water and effluent were brought to 25°C prior to making the dilution series. The temperature of resulting effluent dilution is assumed to also be 25°C.

QA: DEP 5/15/20

CHRONIC CHEMICAL DATA (INITIAL)

QA: DEP 5/19/20

Project Number:	17001-474-069
Test Species: <i>Artemia franciscana</i>	

%	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Meter #	Remarks
Conc.:	150								All Conc.	
pH	7.1	/	7.2	/	/	/	/	/		
D.O. (mg/L)	5.1	/	5.3	/	/	/	/	/		
Temp. (°C)	20	/	20	/	/	/	/	/		
Cond. (µS/cm)	138,800	/	137,000	/	/	/	/	/		
Conc.:		/		/	/	/	/	/		
pH		/		/	/	/	/	/		
D.O. (mg/L)		/		/	/	/	/	/		
Temp. (°C)		/		/	/	/	/	/		
Cond. (µS/cm)		/		/	/	/	/	/		
Conc.:		/		/	/	/	/	/		
pH		/		/	/	/	/	/		
D.O. (mg/L)		/		/	/	/	/	/		
Temp. (°C)		/		/	/	/	/	/		
Cond. (µS/cm)		/		/	/	/	/	/		
Conc.:		/		/	/	/	/	/		
pH		/		/	/	/	/	/		
D.O. (mg/L)		/		/	/	/	/	/		
Temp. (°C)		/		/	/	/	/	/		
Cond. (µS/cm)		/		/	/	/	/	/		
Conc.:	300									
pH	6.9	/	7.0	/	/	/	/	/		
D.O. (mg/L)	5.0	/	5.3	/	/	/	/	/		
Temp. (°C)	20	/	20	/	/	/	/	/		
Cond. (µS/cm)	139,700	/	137,300	/	/	/	/	/		
Hard. (mg/L)		/		/	/	/	/	/		
Alk. (mg/L)		/		/	/	/	/	/		
TRC (mg/L)		/		/	/	/	/	/		
NH ₃ (mg/L)		/		/	/	/	/	/		
Date:	5/14/20		5/16/20							
Time:	1455		1545							
Initials:	CP		UES CP							

Note: Hardness, alkalinity, TRC, and NH₃ data appearing on this page have been transcribed from the wet chemistry log QA Form No. 084.

*Dilution/control water and effluent were brought to 25C prior to making the dilution series. The temperature of resulting effluent dilution is assumed to also be 25C.

UES 5/15/20; WP

CHRONIC CHEMICAL DATA (FINAL)

QA: DP 5/19/20

Project Number:	17001-474-069
Test Species:	<i>Artemia franciscana</i>

%	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Meter #	Remarks
Conc.: 0				126100	/	/	/	/	All Conc.	* conductivity
pH	8.0	8.1	8.2	8.0	/	/	/	/	FM27	
D.O. (mg/L)	7.5	5.6	5.5	5.2	/	/	/	/	17	
Temp (°C)	22	22 ^Δ	20	23 ^Δ	/	/	/	/	L-6	
Conc.: 19					/	/	/	/		* conductivity
pH	8.0	8.1	8.1	7.9	/	/	/	/		
D.O. (mg/L)	7.5	5.6	5.6	5.6	/	/	/	/		
Temp (°C)	22	22 ^Δ	20	23 ^Δ	/	/	/	/		
Conc.: 37.5	-				/	/	/	/		* conductivity
pH	7.9	8.0	8.0	7.9	/	/	/	/		
D.O. (mg/L)	7.6	5.7	5.5	5.2	/	/	/	/		
Temp (°C)	22	22 ^Δ	20	23 ^Δ	/	/	/	/		
Conc.: 75					/	/	/	/		* conductivity
pH	7.7	7.9	7.9	7.8	/	/	/	/		
D.O. (mg/L)	7.6	5.6	5.4	5.4	/	/	/	/		
Temp (°C)	22	22 ^Δ	21	23 ^Δ	/	/	/	/		
Conc.: 150					/	/	/	/		* conductivity
pH	7.5	7.5	7.7	7.6	/	/	/	/		
D.O. (mg/L)	7.3	5.5	5.3	5.5	/	/	/	/		
Temp (°C)	22	22 ^Δ	20	23 ^Δ	/	/	/	/		
Conc.: 300				128600	/	/	/	/		* conductivity
pH	7.1	7.0	7.4	7.4	/	/	/	/		
D.O. (mg/L)	7.8	5.5	5.5	5.4	/	/	/	/		
Temp (°C)	22	22 ^Δ	20	23 ^Δ	/	/	/	/		
Conc.:					/	/	/	/		
pH					/	/	/	/		
D.O. (mg/L)					/	/	/	/		
Temp (°C)					/	/	/	/		
Date:	5/15/20	5/16/20	5/17/20	5/18/20						
Time:	1800	1630		1445						
Initials:	ES	CP	CP	EN						

Δ checked all reps

DAILY TOXICITY TEST LOG

QA: DAP 5/19/20

Project Number:	17001-474-069
Test Species:	<i>Artemia franciscana</i>

General Comments	Feeding	Initials/Date
Random Chart: "P" Min/Max Thermometer # L-29	72.5 ug/l Chla 0.33ml YTC	
Test Day 0 Test Solution Mixed at: 1445 Test Organisms Added at: 1505	Fed @ 1150	CP 5/14/20
Test Day 1 Real Time: 21 °C Min-Max Range: 21- 22 21 °C	NONE	ES/CP 5/15/20
Test Day 2 Real Time: 21 °C Min-Max Range: 21 - 21 °C *Moved test from Bath 2 to Bath 1 due to high temps	Fed @ 1245 CP	CP 5/16/20
Test Day 3 Real Time: 20 °C Min-Max Range: 20 - 22 °C	NONE	CP 5/17/20
Test Day 4 Real Time: 20 °C Min-Max Range: 20 - 22 °C	NONE	EN 5/18/20

05 5/18/20

CETIS Analytical Report

Artemia franciscana

Report Date: 18 May-20 16:33 (p 1 of 3)

Test Code: 474-069 | 13-3467-6586

① Fathead Minnow 96-h Acute Survival Test

TRE Environmental Strategies

Analysis ID: 05-2429-3739	Endpoint: 96h Survival Rate	CETIS Version: CETISv1.8.7
Analyzed: 18 May-20 16:26	Analysis: Linear Regression (MLE)	Official Results: Yes
Batch ID: 01-3349-1304	Test Type: Survival (96h)	Analyst: Lab Tech
Start Date: 14 May-20 15:05	Protocol: EPA/821/R-02-012 (2002)	Diluent: rGSL
Ending Date: 18 May-20 14:50	Species: Artemia franciscana	Brine: Crystal Sea
Duration: 96h	Source: In-House Culture	Age: 48h
Sample ID: 01-6714-1474	Code: 9F66062	Client: Notre Dame
Sample Date: 14 May-20 11:50	Material: Zinc sulfate	Project: Special Studies
Receive Date: 14 May-20 11:50	Source: Discharge Monitoring Report	
Sample Age: 3h	Station: Mock	

Linear Regression Options

Model Function	Threshold Option	Threshold	Optimized	Pooled	Het Corr	Weighted
Log-Normal [NED=A+B*log(X)]	Control Threshold	0.025	Yes	No	No	Yes

Regression Summary

Iters	LL	AICc	BIC	Mu	Sigma	Adj R2	F Stat	Critical	P-Value	Decision(α:5%)
9	-57.44	122.1	124.4	2.095	0.1366	0.8968	0.9945	3.16	0.4178	Non-Significant Lack of Fit ✓

Point Estimates

Level	mg/L ①	95% LCL	95% UCL
LC5	74.27	48.06	91.06
LC10	83.25	57.76	99.38
LC15	89.92	65.3	105.6
LC20	95.61	71.91	110.9
LC25	100.8	78.01	115.8
LC40	115	95.11	130.1
LC50	124.6	106.3	140.7

Regression Parameters

Parameter	Estimate	Std Error	95% LCL	95% UCL	t Stat	P-Value	Decision(α:5%)
Threshold	0.04249	0.01831	0.006595	0.07838	2.32	0.0305	Significant Parameter
Slope	7.323	1.486	4.411	10.23	4.929	<0.0001	Significant Parameter
Intercept	-15.34	3.171	-21.56	-9.13	-4.84	<0.0001	Significant Parameter

ANOVA Table

Source	Sum Squares	Mean Square	DF	F Stat	P-Value	Decision(α:5%)
Model	153.8072	153.8072	1	202	<0.0001	Significant
Lack of Fit	2.273999	0.758	3	0.9945	0.4178	Non-Significant
Pure Error	13.71934	0.762186	18			
Residual	15.99334	0.761588	21			

Residual Analysis

Attribute	Method	Test Stat	Critical	P-Value	Decision(α:5%)
Goodness-of-Fit	Pearson Chi-Sq GOF	15.99	32.67	0.7700	Non-Significant Heterogeneity
	Likelihood Ratio GOF	16.65	32.67	0.7320	Non-Significant Heterogeneity
Variances	Mod Levene Equality of Variance	1.489	2.773	0.2425	Equal Variances
Distribution	Shapiro-Wilk W Normality	0.8833	0.9169	0.0097	Non-normal Distribution
	Anderson-Darling A2 Normality	1.047	2.492	0.0096	Non-normal Distribution

① DAP 5/19/20 E

CETIS Analytical Report

Antonia franciscana

Report Date: 18 May-20 16:33 (p 2 of 3)

Test Code: 474-069 | 13-3467-6586

① ~~Fathead Minnow~~ 96-h Acute Survival Test

TRE Environmental Strategies

Analysis ID: 05-2429-3739 Endpoint: 96h Survival Rate CETIS Version: CETISv1.8.7
 Analyzed: 18 May-20 16:26 Analysis: Linear Regression (MLE) Official Results: Yes

96h Survival Rate Summary

Calculated Variate(A/B)

C-102 ^{ms} ①	Control Type	Count	Mean	Min	Max	Std Err	Std Dev	CV%	%Effect	A	B
0	Dilution Water	4	0.975	0.9	1	0.025	0.05	5.13%	0.0%	39	40
19		4	0.925	0.8	1	0.04787	0.09574	10.4%	5.13%	37	40
37.5		4	0.975	0.9	1	0.025	0.05	5.13%	0.0%	39	40
75		4	0.9	0.8	1	0.04082	0.08165	9.07%	7.69%	36	40
150		4	0.275	0.2	0.4	0.04787	0.09574	34.8%	71.8%	11	40
300		4	0	0	0	0	0		100.0%	0	40

96h Survival Rate Detail

C-102 ^{ms} ①	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Dilution Water	0.9	1	1	1
19		1	1	0.9	0.8
37.5		1	0.9	1	1
75		0.9	0.9	1	0.8
150		0.2	0.2	0.3	0.4
300		0	0	0	0

96h Survival Rate Binomials

C-102 ^{ms} ①	Control Type	Rep 1	Rep 2	Rep 3	Rep 4
0	Dilution Water	9/10	10/10	10/10	10/10
19		10/10	10/10	9/10	8/10
37.5		10/10	9/10	10/10	10/10
75		9/10	9/10	10/10	8/10
150		2/10	2/10	3/10	4/10
300		0/10	0/10	0/10	0/10

① DAP 5/19/20 E

CETIS Analytical Report

① *Artemia franciscana*

Report Date: 18 May-20 16:33 (p 3 of 3)

Test Code: 474-069 | 13-3467-6586

~~Eathead Minnow~~ 96-h Acute Survival Test

TRE Environmental Strategies

Analysis ID: 05-2429-3739

Endpoint: 96h Survival Rate

CETIS Version: CETISv1.8.7

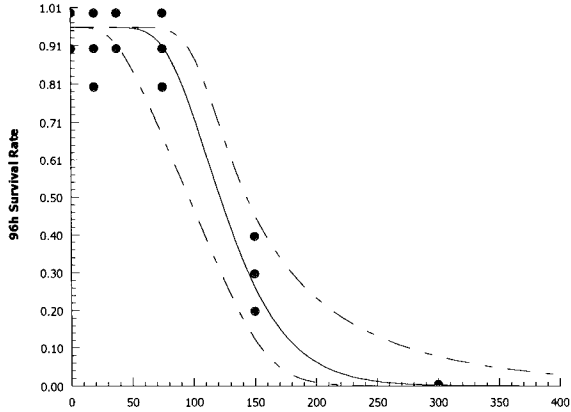
Analyzed: 18 May-20 16:26

Analysis: Linear Regression (MLE)

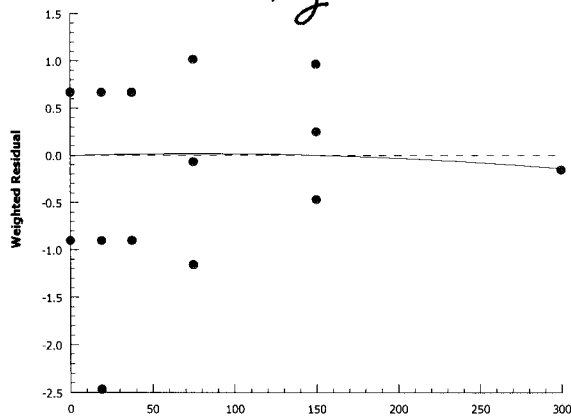
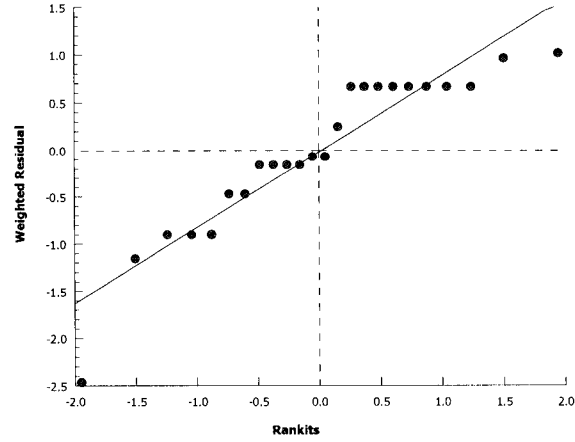
Official Results: Yes

Graphics

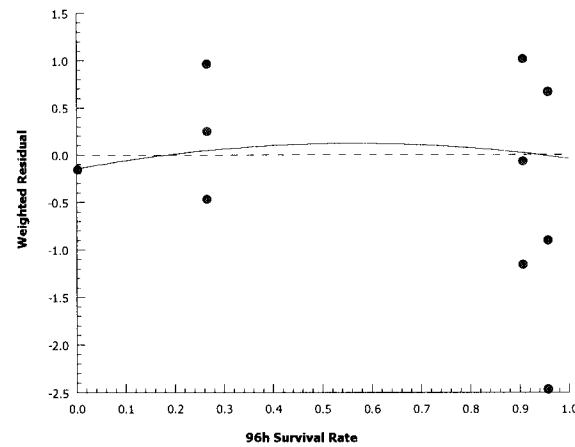
Log-Normal [NED=A+B*log(X)]



C-m/L ①
mg



C-m/L ①
mg



① DAD 5/19/20 E