Bio-Aquatic Testing, Inc.

TCEQ TNI Accredited

City of Nashville
WPCP
OUTFALL 001

Chronic Biomonitoring Report

76521

Ceriodaphnia dubia
Pimephales promelas

September 15, 2020

Approved by: Joshua Reed

Bio-Aquatic Testing, Inc. • 2501 Mayes Rd. Ste. 100 • Carrollton, Texas • 75006

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Unless otherwise noted in the body of the report, all data reported in this document are in compliance with current TNI standards and apply only to the samples referenced within. This report document may not be edited or reproduced in part or in full by any other entity, unless Bio-Aquatic Testing, Inc. issues written approval.

***HAND-WRITTEN RAW DATA TABLES ARE AVAILABLE UPON REQUEST**

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TOXICITY TEST REPORT - Chronic

Client:	Nashville, City of	Sample:	001
Facility:	WPCP	Laboratory Number:	76521
Permit No.	GA0039365	Date:	September 15, 2020

Ceriodaphnia dubia **passed** survival and reproduction testing requirements. *Pimephales promelas* **passed** survival and growth testing requirements.

SAMPLE COLLECTION:

Composite effluent samples from the City of Nashville, WPCP, were received on September 15, 2020 and September 19, 2020. Effluent samples were collected from Outfall 001 by facility personnel. Sample two was received out of hold time and was not used. The test was renewed with sample one until sample three was received. The test was then renewed and completed with sample three.

The effluent samples were analyzed for total residual chlorine using the Hanna Ion Specific Meter #711 and contained <0.10 mg/L and <0.10 mg/L, respectively. Effluent and laboratory dilution water pH, temperature, and dissolved oxygen data were collected daily.

TEST PROCEDURES:

EPA METHOD: 1002

Ceriodaphnia dubia

The seven-day (three brood) Chronic *Ceriodaphnia dubia* survival and reproduction test was initiated at 14:20 hours on September 15, 2020. Five effluent concentrations of 12.5%, 25%, 50%, 69% and 100% were prepared using synthetic water as dilution water. The test was set up with 30mL plastic cups containing 15mL of test solution or control dilution water. Each effluent concentration or control dilution water included ten replicate cups with one organism in each cup. The control was conducted concurrently with the test. Test organisms were less than 24-hour old laboratory cultured neonates. Neonates were introduced into the test solutions using a blocking design. The test was renewed daily with newly prepared solutions. Food consisting of a half-milliliter suspension of the green algae, *Selenastrum capricornutum,* and YTC was added to the test solutions each day. The test proceeded for seven days or until 60% of the females in the control had three broods. Data on survival and number of young produced per female were collected daily. The test ended at 11:03 hours on September 22, 2020. Survival and reproduction data were statistically (p=0.05) analyzed according to EPA procedures to determine the Lowest Observable Effect Concentration (LOEC) and the No Observable Effect Concentration (NOEC).

SURVIVAL:

Ceriodaphnia dubia

Fisher's Exact test on *Ceriodaphnia dubia* survival test data demonstrated no statistically significant differences between the control and any of the effluent concentrations tested.

LOEC: Not Calculable (Q)
NOEC: 100% Effluent

REPRODUCTION:

Ceriodaphnia dubia

The *Ceriodaphnia dubia* reproduction data were normally distributed at the alpha level of 0.01 (13.277) using the Chi-square test for normality. Reproduction data were shown not to be homogeneous using Bartlett's test at the alpha level of 0.01 (15.09) without data transformations. Using the Steel's Many-One Rank Test, *Ceriodaphnia dubia* reproduction data demonstrated no statistically significant differences between the control and any of the effluent concentrations tested.

LOEC: Not Calculable (Q)
NOEC: 100% Effluent

TEST PROCEDURES:

EPA METHOD: 1000

Pimephales promelas

The seven-day Chronic *Pimephales promelas* survival and growth test was initiated at 15:45 hours on September 15, 2020. Five effluent concentrations of 12.5%, 25%, 50%, 69% and 100% were prepared using synthetic water as dilution water. The test was set up with 450mL plastic cups containing 250mL of test solution as test chambers. Each concentration consisted of five replicate chambers containing eight organisms each, giving a total of 40 (forty) per treatment. The control test was conducted concurrently with the test. Test organisms were laboratory-cultured *Pimephales promelas* larvae less than 24-hours old. The number of surviving larvae and water quality parameters in the old test solutions were recorded after each 24-hour period. The test was renewed daily with fresh solutions. Surviving larvae in each test chamber were fed freshly hatched brine shrimp two times per day. The test proceeded for seven days.

At the end of the test, all organisms were sacrificed, dried, and weighed. Data on surviving organisms and water quality were collected. The test ended at 10:06 hours on September 22, 2020. Survival and growth (weight) were statistically (p=0.05) analyzed according to EPA procedures to determine the Lowest Observable Effect Concentration (LOEC) and the No Observable Effect Concentration (NOEC).

SURVIVAL:

Pimephales promelas

ANOVA and Dunnett's Test performed on *Pimephales promelas* survival data demonstrated no statistically significant differences between the control and any of the effluent concentrations tested.

LOEC: Not Calculable (Q)
NOEC: 100% Effluent

GROWTH:

Pimephales promelas

The *Pimephales promelas* growth data were normally distributed at the alpha level of 0.01 (0.900) using Shapiro Wilk's test for normality. Growth data were shown to be homogeneous using Bartlett's test at the alpha level of 0.01 (15.09) without data transformations. Using ANOVA and Dunnett's Test on *Pimephales promelas* growth data demonstrated no statistically significant differences between the control and any of the effluent concentrations tested.

LOEC: Not Calculable (Q)
NOEC: 100% Effluent

BIO-AQUATIC TESTING, INC.

TOXICITY TEST

Chronic ***Ceriodaphnia dubia***

Client: Nashville, City of WPCP

Permit Number: NPDES GA0039365

Sample Type: Composite

Outfall Name: 001

Receiving Water Name:

Lab ID: 76521

Test Temperature (oC): 25 ± 1

Photo Period: 16 hours light, 8 hours dark

Dilution Water: synthetic

Begin Date: 9/15/2020

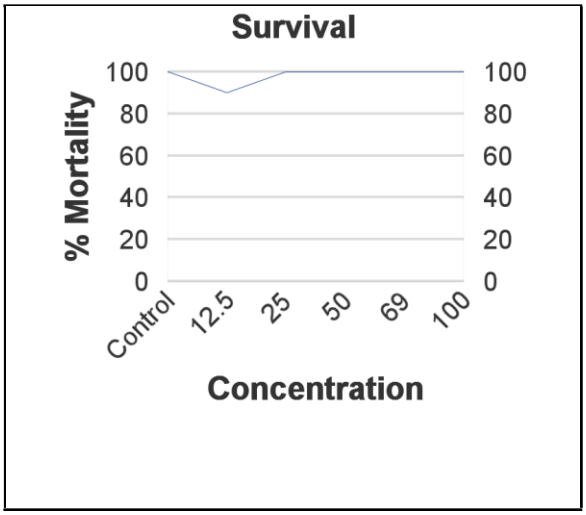
End Date: 9/22/2020

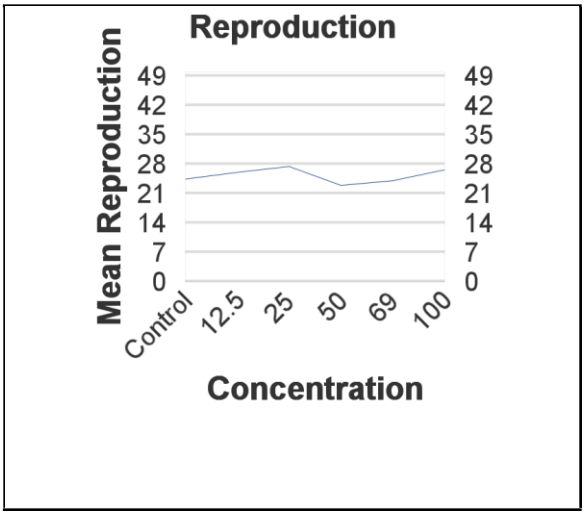
Test Start Time: 14:20 **Test End Time:** 11:03

SURVIVAL AND REPRODUCTION TABLE

FEMALE #	Control	12.5	%	25	%	50	%	69	%	100	%
1	24	24		30		25		28		27	
2	25	26		30		27		25		23	
3	28	23		22		23		25		28	
4	27	31		27		23		21		31	
5	27	25		24		21		25		25	
6	19	33		32		19		19		32	
7	19	26		25		24		25		34	
8	26	21		28		27		29		22	
9	24	25		24		24		20		22	
10	25	D- 0		32		16		23		22	
Surv.Mean	24.4	26.0		27.4		22.9		24.0		26.6	
C.V%	12.8	14.5		13		15		13.6		17	
Total Mean	24.4	23.4		27.4		22.9		24.0		26.6	
Var	9.822	14.25		12.711		11.877		10.666		20.488	
Std.Dev.	3.134	3.774		3.565		3.446		3.265		4.526	
Max	28	33		32		27		29		34	
Min	19	21		22		16		19		22	

Concentration Response Relationships





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Survival and Reproduction

Control

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	3	4	A	A	A	A	A	3	A	A
9/19	A	A	3	4	A	A	1	A	3	5
9/20	10	12	12	9	14	8	8	10	10	8
9/21	11	9	A	A	A	A	A	13	A	A
	24	25	15	13	14	8	9	26	13	13
9/22	A	A	13	14	13	11	10	A	11	12
	24	25	28	27	27	19	19	26	24	25
9/23										

Mean: 24.40 **CV%** 12.80
Var. 9.82 **Max** 28
Std.Dev. 3.13 **Min** 19

12.5

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	4	4	A	A	A	A	A	A	A	A
9/19	A	A	3	5	4	5	5	3	3	D
9/20	7	8	10	13	6	12	5	7	9	D
9/21	13	14	A	A	A	A	A	11	A	D
	24	26	13	18	10	17	10	21	12	0
9/22	A	A	10	13	15	16	16	A	13	D
	24	26	23	31	25	33	26	21	25	0
9/23										

Mean: 26.00 **CV%** 14.50
Var. 14.25 **Max** 33
Std.Dev. 3.77 **Min** 21

25

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	3	5	A	A	A	A	A	4	A	A
9/19	A	A	5	4	3	6	A	A	5	4
9/20	11	10	10	12	7	8	8	10	7	9
9/21	16	15	A	A	A	A	A	A	A	A
	30	30	15	16	10	14	8	14	12	13
9/22	A	A	7	11	14	18	17	14	12	19
	30	30	22	27	24	32	25	28	24	32
9/23										

Mean: 27.40 **CV%** 13.00
Var. 12.71 **Max** 32
Std.Dev. 3.57 **Min** 22

50

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	A	3	A	A	A	A	A	4	A	A
9/19	1	A	4	5	6	4	5	A	3	4
9/20	9	11	10	10	6	7	6	11	9	7
9/21	15	13	A	A	A	A	A	12	A	A
	25	27	14	15	12	11	11	27	12	11
9/22	A	A	9	8	9	8	13	A	12	5
	25	27	23	23	21	19	24	27	24	16
9/23										

Mean: 22.90 **CV%** 15.00
Var. 11.88 **Max** 27
Std.Dev. 3.45 **Min** 16

69

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	3	4	A	A	A	A	A	4	A	A
9/19	A	A	3	4	4	A	5	A	2	3
9/20	7	8	10	10	7	9	8	11	7	7
9/21	18	13	A	A	A	A	A	14	A	A
	28	25	13	14	11	9	13	29	9	10
9/22	A	A	12	7	14	10	12	A	11	13
	28	25	25	21	25	19	25	29	20	23
9/23										

Mean: 24.00 **CV%** 13.60
Var. 10.67 **Max** 29
Std.Dev. 3.27 **Min** 19

100

Date	1	2	3	4	5	6	7	8	9	10
9/16	A	A	A	A	A	A	A	A	A	A
9/17	A	A	A	A	A	A	A	A	A	A
9/18	3	A	A	A	A	A	5	3	A	A
9/19	A	A	4	3	A	2	A	A	A	4
9/20	9	9	10	12	7	10	11	8	8	4
9/21	15	14	A	A	A	A	A	11	A	A
	27	23	14	15	7	12	16	22	8	8
9/22	A	A	14	16	18	20	18	A	14	14
	27	23	28	31	25	32	34	22	22	22
9/23										

Mean: 26.60 **CV%** 17.00
Var. 20.49 **Max** 34
Std.Dev. 4.53 **Min** 22

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Chronic CERIODAPHNIA DUBIA SURVIVAL AND REPRODUCTION

Client: **Nashville, City of - WPCP** Lab ID: **76521** Culture No.: B609082a

TEST INSTRUCTIONS:

ORGANISMS ADDED: Date: 9-15-20 Time: 1420 Technician: SC

Photo Period 16hr Light/8hr dark

RANDOMIZATION:

SC-10 27

Dilution: Control

	DATE/TIME/ TECHNICIAN	1	2	3	4	5	6	7	8	9	10
24Hr	9-16-20 MC1058	A									
48Hr	9-17-20 MC1015	A									
72Hr	9-18-20 1646SW	3	4	A	A	A	A	A	3	A	A
96Hr	9-19-20 1338SW	A	A	3	4	A	A	1/A	3	3	5
5 days	9-20-20 1625SW	10	12	12	9	14	8	8	7	10	8
6 days	9-21-20 MC1130	11	9	A					13	A	A
7 days	9-22-20 1103SW	A	A	13	14	13	11	10	A	11	12
8 days											

Dilution: 12.5 %

	1	2	3	4	5	6	7	8	9	10
24Hr	A									
48Hr	A									
72Hr	4	4	A	A	A	A	A	A	A	A
96Hr	A	A	3	5	4	5	5	3/A	3	D
5 days	9	8	10	13	6	12	5	1/9	9	
6 days	13	14	A					11	A	
7 days	A	A	10	12	15	16	16	A	12	
8 days										

Code: Cells in numbered columns indicate daily survival and reproduction: "A" means adult alive and no young produced, a number means adult alive and that number of young produced, "D" followed by a zero means adult dead and no young produced, "D" followed by a number means adult dead and that number of young produced. "E" indicates toss out due to experimenter error. Lined through spaces preceded by a number or letter represent the same number. Lined spaces without a preceding number or letter indicate unused or not applicable spaces.

BIO-AQUATIC TESTING, INC.

Chronic CERIODAPHNIA DUBIA SURVIVAL AND REPRODUCTION

Client: **Nashville, City of - - WPCP** Lab ID: **76521** Culture No.:

TEST INSTRUCTIONS:

Dilution: 25 %

	1	2	3	4	5	6	7	8	9	10
24Hr	A									
48Hr	A									
72Hr	3	5	A					4	A	A
96Hr	A	A	5	4	3	6	A	A	5	4
5 days	11	10	10	12	7	8	8	10	7	9
6 days	16	15	A					11	A	A
7 days	A	A	7	11	14	18	17	3	12	19
8 days										

Dilution: 50 %

	1	2	3	4	5	6	7	8	9	10
24Hr	A									
48Hr	A									
72Hr	A	3	A					4	A	A
96Hr	1/A	A	4	5	6	4	5	A	3	1/3
5 days	9	11	10	10	6	7	6	11	9	7
6 days	15	13	A					12	A	A
7 days	A	A	9	8	9	8	13	A	12	5
8 days										

Code: Cells in numbered columns indicate daily survival and reproduction: "A" means adult alive and no young produced, a number means adult alive and that number of young produced, "D" followed by a zero means adult dead and no young produced, "D" followed by a number means adult dead and that number of young produced. "E" indicates toss out due to experimenter error. Lined through spaces preceded by a number or letter represent the same number. Lined spaces without a preceding number or letter indicate unused or not applicable spaces.

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Chronic CERIODAPHNIA DUBIA SURVIVAL AND REPRODUCTION

Client: **Nashville, City of - WPCP** Lab ID: **76521** Culture No.:

TEST INSTRUCTIONS:

Dilution: 69 %

	1	2	3	4	5	6	7	8	9	10
24Hr	A									
48Hr	A									
72Hr	3	4	A					4	A	A
96Hr	A	A	3	4	4	A	5	A	3/A	3
5 days	7	8	10	10	7	9	8	11	7	7
6 days	18	13	A					14	A	A
7 days	A	A	12	7	14	10	12	A	11	13
8 days										

Dilution: 100 %

	1	2	3	4	5	6	7	8	9	10
24Hr	A									
48Hr	A									
72Hr	3	A	A	A	A	A	5	3	A	A
96Hr	A	A	4	3	A	3/A	A	A	A	4
5 days	9	9	10	12	7	10	11	9	8	4
6 days	15	14	A					11	A	A
7 days	A	A	14	16	18	20	18	A	14	14
8 days										

Code: Cells in numbered columns indicate daily survival and reproduction: "A" means adult alive and no young produced, a number means adult alive and that number of young produced, "D" followed by a zero means adult dead and no young produced, "D" followed by a number means adult dead and that number of young produced. "E" indicates toss out due to experimenter error. Lined through spaces preceded by a number or letter represent the same number. Lined spaces without a preceding number or letter indicate unused or not applicable spaces.

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BIO-AQUATIC TESTING, INC.

Chronic CERIODAPHNIA DUBIA SURVIVAL AND REPRODUCTION

Client: **Nashville, City of - WPCP** Lab ID: **76521** Culture No.:

TEST INSTRUCTIONS:

Test Temperatures

	0Hr	24Hr		48Hr		72Hr		96Hr		5 days		6 days		7 days
	new	old	new	old	new	old	new	old	new	old	new	old	new	old
Control	25.7	25.1	25.1	25.3	25.8	25.2	25.2	25.2	25.3	25.2	25.0	25.1	25.3	25.2
12.5														
25														
50														
69														
100														
TIME/DATE TECH	9-15-20 SW 1420	9-16-20 MC 1058		9-17-20 MC 1015		9-18-20 1696 JW		9-19-20 1330 JW		9-20-20 1625 JW		9-21-20 MC 1130		9-22-20 1103 JW
IR GUN ID #	018	018		018		012		012		012		018		012

Lined through spaces preceded by a number represent the same number. Lined spaces without a preceding number indicate unused or not applicable spaces.

Chronic *Pimephales promelas*

Client: Nashville, City of WPCP
Lab ID: 76521
Permit Number: NPDES GA0039365
Test Temperature (oC): 25 ± 1
Outfall Name: 001
Sample Type: Composite
Photo Period: 16 Hours Light 8 Hours Dark
Receiving Water Name:

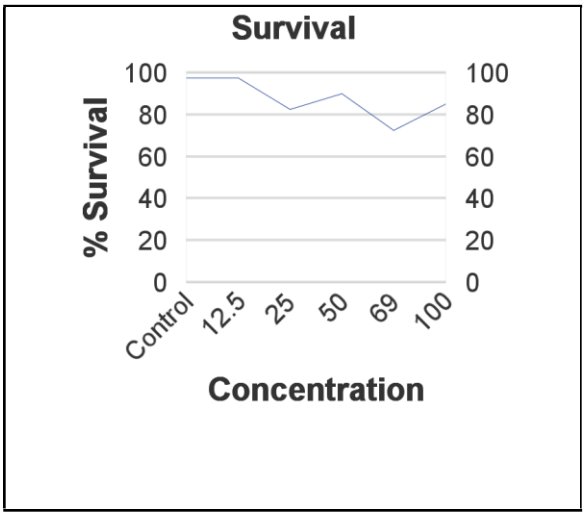
Test Start Time: 15:45 Test End Time: 10:06
Begin Date: 9/15/2020
End Date: 9/22/2020

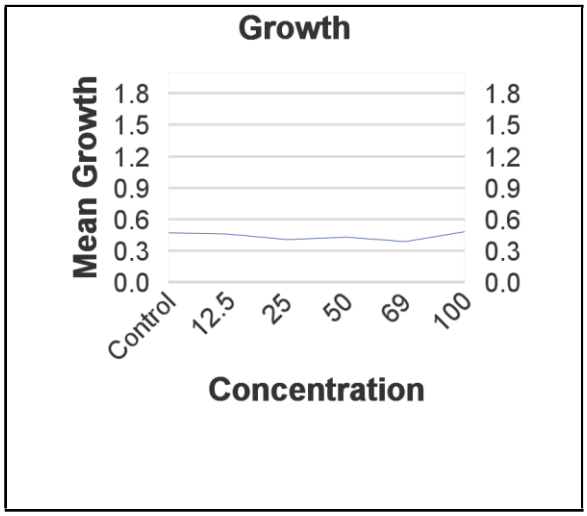
SURVIVAL

Effluent Concentration		Number Of Alive 9/15	9/16	9/17	9/18	9/19	9/20	9/21	9/22	Avg% Surv.
Control	A	8	8	7	7	7	7	7	7	97.5%
	B	8	8	8	8	8	8	8	8	
	C	8	8	8	8	8	8	8	8	
	D	8	8	8	8	8	8	8	8	
	E	8	8	8	8	8	8	8	8	
12.5	A	8	8	8	8	8	8	8	8	97.5%
	B	8	8	8	8	8	8	8	8	
	C	8	8	8	8	8	8	8	8	
	D	8	8	8	8	8	7	7	7	
	E	8	8	8	8	8	8	8	8	
25	A	8	8	8	7	7	6	6	6	82.5%
	B	8	8	8	8	8	8	8	8	
	C	8	8	8	8	8	8	8	8	
	D	8	8	8	8	8	8	8	8	
	E	8	8	8	5	4	3	3	3	
50	A	8	8	8	8	8	8	8	8	90.0%
	B	8	8	8	8	8	8	8	7	
	C	8	8	8	8	6	5	5	5	
	D	8	8	8	8	8	8	8	8	
	E	8	8	8	8	8	8	8	8	

Effluent Concentration		Number Of Alive 9/15	9/16	9/17	9/18	9/19	9/20	9/21	9/22	Avg% Surv.
69	A	8	8	8	7	7	5	3	3	72.5%
	B	8	8	8	8	7	6	6	6	
	C	8	8	8	6	6	6	5	5	
	D	8	8	8	7	7	7	7	7	
	E	8	8	8	8	8	8	8	8	
100	A	8	8	7	7	7	7	7	7	85.0%
	B	8	8	8	8	8	8	8	8	
	C	8	8	8	8	7	5	5	5	
	D	8	8	8	8	8	8	8	8	
	E	8	8	8	8	7	6	6	6	
	A									
	B									
	C									
	D									
	E									

Concentration Response Relationships





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Chronic Pimephales promelas SURVIVAL Lab ID: **76521**

Client: **Nashville, City of** Facility **WPCP** Outfall:001 Sample Type Composite

TEST INSTRUCTIONS:

Culture No. : PI-20-258A Photo Period: 16hr light, 8hr dark **RANDOMIZATION:** **SC-5 2**

Dilution: Control 12.5 25 50

DATE/TIME/ TECHNICIAN	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
0Hr 9-15-20 JM1515	8	—	—	—	—	8	—	—	—	—	8	—	—	—	—	8	—	—	—	—
24Hr 9-16-20 EH0951	8	—	—	—	—	8	—	—	—	—	8	—	—	—	—	8	—	—	—	—
48Hr 9/17/20 US 1158	7	8	—	—	—	8	—	—	—	—	8	—	—	—	—	8	—	—	—	—
72Hr 9-18-20 DT 1324	7	8	—	—	—	8	—	—	—	—	7$_0$	8	—	—	5$_3$	8	—	—	—	—
96Hr 9-19-20 EH1131	7	8	—	—	—	8	—	—	—	—	7	8	—	—	4$_1$	8	8	6$_2$	8	8
5 days 9-20-20 EH1031	7	8	—	—	—	8	—	—	7$_1$	8	6$_1$	8	—	—	3$_1$	8	8	5$_1$	8	8
6 days 9-21-20 EH1047	7	8	—	—	—	8	—	—	7	8	6	8	—	—	3	8	8	5	8	8
7 days 9-22-20 EH1006	7	8	—	—	—	8	—	—	7	8	6	8	—	—	3	8	7$_1$	5	8	8

Dilution: 69 100

	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
0Hr	8	—	—	—	—	8	—	—	—	—										
24Hr	8	—	—	—	—	8	—	—	—	—										
48Hr	8	—	—	—	—	7$_1$	8	—	—	—										
72Hr	7$_1$	8	6$_2$	7$_1$	8	7	8	—	—	—										
96Hr	7	7$_1$	6	7	8	7	8	7$_1$	8	7$_0$										
5 days	5$_2$	6$_1$	6	7	8	7	8	5$_2$	8	6$_1$										
6 days	3$_2$	6	5$_1$	7	8	7	8	5	8	6										
7 days	3	6	5	7	8	7	8	5	8	6										

Lined through spaces preceded by a number represent the same number. Lined spaces without a preceding number indicate unused or not applicable s

BIO-AQUATIC TESTING, INC.

Chronic | Pimephales promelas SURVIVAL | Lab ID: **76521**

Client: **Nashville, City of** | Facility **WPCP** | Outfall:001 | Sample Type: Composite

TEST INSTRUCTIONS:

Test Temperatures

	0Hr	24Hr		48Hr		72Hr		96Hr		5 days		6 days		7 days
	new	old	new	old	new	old	new	old	new	old	new	old	new	old
Control	25.3	25.1	24.9	25.5	25.2	25.6	25.6	24.2	24.9	25.1	25.5	24.9	25.1	24.8
12.5														
25														
50											25.6			
69														
100														
TIME/DATE TECH	9-15-20 JW 1545	9-16-20 EH	0952	9-17-20	1158	9-17-20 1324	DT	9-19-20 EH	1132	9-20-20 EH	1032	9-21-20 EH	1048	9-22-20 EH 1007
IR GUN ID #	020	020		020		020		020		020		020		020

Lined through spaces preceded by a number represent the same number. Lined spaces wilout a preceding number indicate unused or not applicable spaces.

2nd Organism **BIO-AQUATIC TESTING, INC.** **TOXICITY TEST**

Chronic *Pimephales promelas*

Client: Nashville, City of WPCP **Lab ID: 76521**

Permit Number: GA0039365

Sample Type: Composite **Outfall Name:** 001

Receiving Water Name:

Synthetic

	ON	SN	Wt.	Avg.	SN Avg.
A	8	7	4.086	0.511	0.584
B	8	8	3.985	0.498	0.498
C	8	8	3.626	0.453	0.453
D	8	8	3.050	0.381	0.381
E	8	8	4.158	0.520	0.520

Mean	C.V. %
0.473	12.1

SN Mean	SN C.V. %
0.487	15.5

12.5

	ON	Wt.	Avg.
A	8	3.824	0.478
B	8	3.872	0.484
C	8	3.648	0.456
D	8	3.664	0.458
E	8	3.421	0.428

Mean	C.V. %
0.461	4.8

25

	ON	Wt.	Avg.
A	8	3.720	0.465
B	8	3.473	0.434
C	8	3.453	0.432
D	8	3.759	0.470
E	8	1.930	0.241

Mean	C.V. %
0.408	23.3

50

	ON	Wt.	Avg.
A	8	3.422	0.428
B	8	3.634	0.454
C	8	2.375	0.297
D	8	4.589	0.574
E	8	3.214	0.402

Mean	C.V. %
0.431	23.1

69

	ON	Wt.	Avg.
A	8	1.713	0.214
B	8	3.218	0.402
C	8	3.132	0.392
D	8	3.345	0.418
E	8	4.161	0.520

Mean	C.V. %
0.389	28.4

100

	ON	Wt.	Avg.
A	8	4.659	0.582
B	8	4.007	0.501
C	8	3.015	0.377
D	8	4.307	0.538
E	8	3.329	0.416

Mean	C.V. %
0.483	17.6

	ON	Wt.	Avg.
A			
B			
C			
D			
E			

Mean	C.V. %

	ON	Wt.	Avg.
A			
B			
C			
D			
E			

Mean	C.V. %

Note: ON stands for original number per replicate, while SN refers to the number surviving after test completion.

BIO-AQUATIC TESTING, INC. TOXICITY TEST

Chronic — Pimephales promelas — Lab ID: 76521

Client: Nashville, City of - WPCP

Balance: Radwag BAL-007

Begin Date: 9/15/2020 End Date: 9/22/2020 Organism: Pimephales promelas

Analyst: BA

Weigh Date: 09/24/20

Date/Time placed in Oven: 09/22/20 | 1415

Date/Time removed from Oven: 09/23/20 | 1415

Control

	Qty.	Wt.
A	7	4.086
B	8	3.985
C		3.626
D		3.050
E		4.158

12.5 %

	Qty.	Wt.
A	8	3.824
B		3.872
C		3.648
D	7	3.664
E	8	3.421

25 %

	Qty.	Wt.
A	6	3.720
B	8	3.473
C		3.453
D		3.759
E	3	1.930

50 %

	Qty.	Wt.
A	8	3.422
B	7	3.634
C	5	2.375
D	8	4.589
E	8	3.214

69 %

	Qty.	Wt.
A	3	1.713
B	6	3.218
C	5	3.132
D	7	3.345
E	8	4.161

100 %

	Qty.	Wt.
A	7	4.659
B	8	4.007
C	5	3.015
D	8	4.307
E	6	3.329

	Qty.	Wt.
A		
B		
C		
D		
E		

	Qty.	Wt.
A		
B		
C		
D		
E		

	Qty.	Wt.
A		
B		
C		
D		
E		

Lined through spaces preceded by a number represent the same number. Lined spaces without a preceding number indicate unused or not applicable spaces.

APPENDIX A

STATISTICS SUMMARY

Both the lethal and sub-lethal endpoints were statistically calculated according to their respective EPA guidelines. The Chronic Freshwater organisms were calculated according to EPA-821-R-02-013, October 2002 Fourth Edition. The Chronic Marine and Estuarine organisms were calculated according to EPA-821-R-02-014, October 2002 Third Edition. The Acute Freshwater and Marine organisms were calculated according to EPA-821-R-02-012, October 2002 Fifth Edition. The fertilization organisms were calculated according to EPA-600-R-95-136 or EPA-600-R-12-022, dependent upon the species. Listed below are the basic principles of these guidelines. If you would like a copy of the raw statistical calculations for your test then please contact us.

The chronic and acute *Pimephales promelas* and *Menidia beryllina* survival data is analyzed using Shapiro Wilks Test and Bartlett's Test. If the data passes both tests then the data is run through ANOVA and Dunnetts (parametric). If the data fails Shapiro Wilks Test or Bartlett's Test then Steels Many One Test (non-parametric) is used. The chronic *Pimephales promelas* and *Menidia beryllina* growth data is analyzed using Shapiro Wilks Test and Bartlett's Test. If the data passes one of these tests then the data is run through ANOVA and Dunnetts. If the data fails Shipiro Wilks Test and Bartlett's Test then Steels Many One Test is used. Point estimation may also be used.

The chronic *Mysidopsis bahia* survival data is analyzed using Chi-square test and Bartlett's Test. If the data passes both tests then the data is run through ANOVA and Dunnetts. If the data fails Chi-square test or Bartlett's Test then Steels Many One Test is used. *Mysidopsis bahia* growth data is analyzed using Chi-square test and Bartlett's Test. If the data passes one of these tests then the data is run through ANOVA and Dunnetts. If the data fails Chi-square test and Bartlett's Test then Steels Many One Test is used. Point estimation may also be used.

The acute *Mysidopsis bahia* survival data is analyzed using Shapiro Wilks Test and Bartlett's Test. If the data passes both tests then the data is run through ANOVA and Dunnetts. If the data fails Shipiro Wilks Test or Bartlett's Test then Steels Many One Test is used. Point estimation may also be used.

The chronic *Ceriodaphnia dubia* survival data are analyzed using the Fisher's Exact Test. The chronic *Ceriodaphnia dubia* reproduction and are analyzed using the Chi-square test and Bartlett Test. If the data passes one of these tests then the data is run through ANOVA and Dunnetts. If the data fails Chi-square test and Bartlett's Test then Steels Many One Test is used. Point estimation may also be used.

The acute *Daphnia pulex* and *Ceriodaphnia dubia* survival data is analyzed using Shapiro Wilks Test and Bartlett's Test. If the data passes both tests then the data is run through ANOVA and Dunnetts. If the data fails Shapiro Wilks Test or Bartlett's Test then Steels Many One Test is used. Point estimation may also be used.

The fertilization data is analyzed using Shapiro Wilks Test and Bartlett's Test. If the data passes both tests then the data is run through ANOVA and Dunnetts. If the data fails Shapiro Wilks Test or Bartlett's Test then Steels Many One Test is used. Point estimation or TST methodology may also be used.

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	4.020	14.520	22.920	14.520	4.020
OBSERVED	6	11	25	16	2

Calculated Chi-Square goodness of fit test statistic = 3.1832
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

Bartlett's test for homogeneity of variance
Calculated B1 statistic = 17.86

Table Chi-square value = 15.09 (alpha = 0.01, df = 5)
Table Chi-square value = 11.07 (alpha = 0.05, df = 5)

Data FAIL B1 homogeneity test at 0.01 level. Try another transformation.

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	163.683	32.737	1.347
Within (Error)	54	1312.500	24.306	
Total	59	1476.183		

Critical F value = 2.45 (0.05,5,40)
Since F < Critical F FAIL TO REJECT Ho: All equal

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	con	24.400	24.400		

2	12.5	23.400	23.400	0.454
3	25	27.400	27.400	-1.361
4	50	22.900	22.900	0.680
5	69	24.000	24.000	0.181
6	100	26.600	26.600	-0.998

Dunnett table value = 2.31 (1 Tailed Value, P=0.05, df=40,5)

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	con	10			
2	12.5	10	5.093	20.9	1.000
3	25	10	5.093	20.9	-3.000
4	50	10	5.093	20.9	1.500
5	69	10	5.093	20.9	0.400
6	100	10	5.093	20.9	-2.200

cerio repro
File: 76521.cdr Transform: NO TRANSFORMATION

STEEL'S MANY-ONE RANK TEST - Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	RANK SUM	CRIT. VALUE	df	SIG
1	con	24.400				
2	12.5	23.400	105.00	75.00	10.00	
3	25	27.400	125.50	75.00	10.00	
4	50	22.900	89.00	75.00	10.00	
5	69	24.000	101.50	75.00	10.00	
6	100	26.600	115.50	75.00	10.00	

Critical values use k = 5, are 1 tailed, and alpha = 0.05

fathead survival
File: 76521.pps Transform: NO TRANSFORMATION

Shapiro - Wilk's test for normality

D = 49.200

W = 0.911

Critical W (P = 0.05) (n = 30) = 0.927
Critical W (P = 0.01) (n = 30) = 0.900

Data PASS normality test at P=0.01 level. Continue analysis.

fathead survival
File: 76521.pps Transform: NO TRANSFORMATION

Bartlett's test for homogeneity of variance
Calculated B1 statistic = 13.08

Table Chi-square value = 15.09 (alpha = 0.01, df = 5)
Table Chi-square value = 11.07 (alpha = 0.05, df = 5)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

fathead survival
File: 76521.pps Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	14.800	2.960	1.444
Within (Error)	24	49.200	2.050	
Total	29	64.000		

Critical F value = 2.62 (0.05,5,24)
Since F < Critical F FAIL TO REJECT Ho: All equal

fathead survival
File: 76521.pps Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	con	7.800	7.800		
2	12.5	7.800	7.800	0.000	
3	25	6.600	6.600	1.325	
4	50	7.200	7.200	0.663	
5	69	5.800	5.800	2.209	
6	100	6.800	6.800	1.104	

Dunnett table value = 2.36 (1 Tailed Value, P=0.05, df=24,5)

fathead survival
File: 76521.pps Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	con	5			

76521

2	12.5	5	2.137	27.4	0.000
3	25	5	2.137	27.4	1.200
4	50	5	2.137	27.4	0.600
5	69	5	2.137	27.4	2.000
6	100	5	2.137	27.4	1.000

fathead growth
File: 76521.ppg Transform: NO TRANSFORMATION

Shapiro - Wilk's test for normality

D = 0.169

W = 0.931

Critical W (P = 0.05) (n = 30) = 0.927
Critical W (P = 0.01) (n = 30) = 0.900

Data PASS normality test at P=0.01 level. Continue analysis.

fathead growth
File: 76521.ppg Transform: NO TRANSFORMATION

Bartlett's test for homogeneity of variance
Calculated B1 statistic = 8.23

Table Chi-square value = 15.09 (alpha = 0.01, df = 5)
Table Chi-square value = 11.07 (alpha = 0.05, df = 5)

Data PASS B1 homogeneity test at 0.01 level. Continue analysis.

fathead growth
File: 76521.ppg Transform: NO TRANSFORMATION

ANOVA TABLE

SOURCE	DF	SS	MS	F
Between	5	0.035	0.007	0.993
Within (Error)	24	0.169	0.007	
Total	29	0.204		

Critical F value = 2.62 (0.05,5,24)
Since F < Critical F FAIL TO REJECT Ho: All equal

fathead growth
File: 76521.ppg Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	con	0.473	0.473		
2	12.5	0.461	0.461	0.222	
3	25	0.408	0.408	1.210	
4	50	0.431	0.431	0.784	
5	69	0.389	0.389	1.572	
6	100	0.483	0.483	-0.192	

Dunnett table value = 2.36 (1 Tailed Value, P=0.05, df=24,5)

fathead growth
File: 76521.ppg Transform: NO TRANSFORMATION

DUNNETT'S TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	con	5			
2	12.5	5	0.125	26.5	0.012
3	25	5	0.125	26.5	0.064
4	50	5	0.125	26.5	0.042
5	69	5	0.125	26.5	0.083
6	100	5	0.125	26.5	-0.010

Bio-Aquatic Testing, Inc.

FRESH WATER TEST SETUP FORM

Client: Nashville, City of

Permit GA0039365

Facility: WPCP

Lab Number 76521

Outfall Name: 001

Number of samples 2

Dilution Water: Synthetic Lab

Receiving Water Name:

Dechlorinate Sample: No

Sx #	Rcvd Date	Rcvd Time	Sampling Dates Begin Date	Sampling Dates End Date	Sampling Times Start	Sampling Times End
1	09/15/20	13:30	09/13/20	09/14/20	09:30	08:00
2	09/19/20	11:33	09/17/20	09/18/20	09:00	07:30

Type of Test(s)

Ceriodaphnia dubia	Chronic
Pimephales promelas	Chronic

Dilution Water

Sample #	Hardness As mg/L $CaCO_3$	Alkalinity as mg/L $CaCO_3$
1	146	61
2	146	60

Start Sx #	1	Date:	9/15/2020
Renew Sx #	1	Date:	9/16/2020
Renew Sx #	1	Date:	9/17/2020
Renew Sx #	1	Date:	9/18/2020
Renew Sx #	1/2	Date:	9/19/2020
Renew Sx #	2	Date:	9/20/2020
Renew Sx #	2	Date:	9/21/2020

Test Start Date: 9/15/2020

Test End Date: 9/22/2020

Ceriodaphnia dubia Test Set Up: 10 Reps & 1 Organisms per Rep

Pimephales Test Set Up: 5 Reps & 8 Organism per Rep

Concentrations: 12.5 25 50 69 100 % LF % 69

Test Chemistry on these dilutions: 12.5 25 50 69 100

Samples received by:

○ Greyhound	○ UPS Next Day	○ Delta Dash	○ Delta
○ Pony Express	○ Client Delivered	○ Southwest Airlines	○ DHL
◉ Federal Express	○ American Airlines	○ Bio Pick Up	

Other:

BIO-AQUATIC TESTING, INC.

Hardness, Alkalinity, Residual Chlorine, Specific Conductivity, and Salinity Analysis Data

Client: Nashville, City of **Lab ID:** 76521

Facility: WPCP **Outfall:** 001

Dilution Water(s): Synthetic Lab **Test Date:** September 15, 2020

EFFLUENT PARAMETERS

Effluent Sample #	Received		Residual Cl_2 (mg/L)	DeChlor (ml/L)[1]	Ammonia (mg/L)	Analyst Initials	Temp. Received
	Date	Time					
1	9/15/20	13:30	<0.10	N/A	<0.25	DF	3.1
2	9/19/20	11:33	<0.10	N/A	<0.25	GS	4.5

[1]**Dechlorination Reagent:** 0.025 N Sodium Thiosulfate

Effluent Sample #	pH	DO (mg/L)	Hardness (mg/L $CaCO_3$)	Alkalinity (mg/L $CaCO_3$)	Conductivity (umhos/cm)	Analyst Initials
1	7.7	9.1	60	56	286	DF
2	7.1	7.9	69	52	209	GS

DAILY RENEWAL CONDUCTIVITY**

Date		Sample #	Values are at Highest Dilution		Analyst
			Specific Conductivity as umhos/cm	Salinity (ppt)	
9/15	Lab H2O		428	0.2	GS
9/16	Lab H2O		439	0.3	JLM/E
9/17	Lab H2O		393	0.2	JLM/G
9/18	Lab H2O		367	0.2	DT
9/19	Lab H2O		379	0.2	EH
9/20	Lab H2O		381	0.2	GS
9/21	Lab H2O		383	0.2	GS
9/15	OUTFALL*	1	304	0.2	GS
9/16	OUTFALL*	1	297	0.2	JLM/E
9/17	OUTFALL*	1	301	0.2	JLM/G
9/18	OUTFALL*	1	303	0.2	DT
9/19	OUTFALL*	1/2	222	0.1	EH
9/20	OUTFALL*	2	221	0.1	GS
9/21	OUTFALL*	2	225	0.1	GS

**Conductivity is taken on the highest remaining effluent concentration used for test renewal, not necessarily 100%

Analysis Methods: Chlorine: Hanna Colorimeter #HI711, Ammonia: Hanna Colorimeter #HI733, Hardness: Hanna Photometer #HI96735, Alkalinity: Hanna Colorimeter #HI775, pH, DO, Conductivity: Thermo Versa Star Benchtop Meter

BIO-AQUATIC TESTING, INC.

pH, Dissolved Oxygen

Chronic **Ceriodaphnia dubia**

Client: Nashville, City of

Lab ID: 76521

Facility: WPCP

Outfall: 001

Dilution Water(s): Synthetic Lab

Test Begin Date: September 15, 2020

NR indicates that the test is non-renewal.

					Concentration							
ANALYST	DATE	TIME	SX#	UNIT	Control	12.5	25	50	69	100		
GS	9/15	Start	1	pH	8.1	8.1	8.0	8.0	7.9	7.9		
		25 ± 1		DO (mg/L)	8.3	8.3	8.3	8.3	8.5	8.5		
JLM/EH	9/16	24 Hr	1	pH	8.0	8.0	8.0	8.0	8.0	8.0		
		25 ± 1		DO (mg/L)	8.2	8.2	8.2	8.3	8.3	8.2		
		Renew	1	pH	8.1	8.0	8.0	7.9	7.9	7.8		
				DO (mg/L)	7.8	8.2	8.2	8.3	8.3	8.6		
JLM/GS	9/17	48 Hr	1	pH	7.9	7.8	7.8	7.8	7.8	7.8		
		25 ± 1		DO (mg/L)	8.3	8.2	8.2	8.2	8.1	8.1		
		Renew	1	pH	8.0	8.0	8.0	8.0	8.0	7.9		
				DO (mg/L)	7.5	7.6	7.6	7.7	7.7	8.1		
DT	9/18	72 Hr	1	pH	7.9	7.9	7.9	7.9	7.9	7.9		
		25 ± 1		DO (mg/L)	8.0	8.0	8.0	7.9	7.9	8.0		
		Renew	1	pH	8.0	8.1	8.1	8.0	8.0	8.0		
				DO (mg/L)	8.0	8.1	8.1	8.3	8.3	8.4		
EH	9/19	96 Hr	1	pH	7.8	7.8	7.8	7.8	7.8	7.8		
		25 ± 1		DO (mg/L)	8.2	8.1	8.1	8.1	8.1	8.1		
		Renew	1/2	pH	7.7	7.7	7.7	7.7	7.7	7.4		
				DO (mg/L)	7.9	8.0	8.0	8.1	8.1	8.5		
GS	9/20	120 Hr	1/2	pH	7.9	7.9	7.9	7.9	7.9	7.9		
		25 ± 1		DO (mg/L)	8.5	8.4	8.5	8.5	8.5	8.5		
		Renew	2	pH	8.0	8.0	7.8	7.8	7.7	7.7		
				DO (mg/L)	8.3	8.3	8.4	8.4	8.5	8.5		
DT/EH	9/21	144 Hr	2	pH	8.1	8.0	8.0	8.0	8.0	8.0		
		25 ± 1		DO (mg/L)	8.2	8.1	8.1	8.2	8.2	8.2		
		Renew	2	pH	7.7	7.6	7.7	7.6	7.6	7.6		
				DO (mg/L)	8.0	8.5	8.5	8.5	8.5	8.5		
GS	9/22	168 Hr	2	pH	7.9	7.9	7.8	7.8	7.8	7.8		
		25 ± 1		DO (mg/L)	8.1	8.0	8.0	8.0	7.9	7.9		

BIO-AQUATIC TESTING, INC.

pH, Dissolved Oxygen

Chronic **Pimephales promelas**

Client: Nashville, City of **Lab Number:** 76521

Facility: WPCP **Dilution Water(s):** Synthetic Lab

Outfall: 001 **Test Begin Date:** September 15, 2020

NR indicates that the test is non-renewal.

					Concentration							
ANALYST	DATE	TIME	SX#	UNIT	Control	12.5	25	50	69	100		
GS	9/15	Start	1	pH	8.1	8.1	8.0	8.0	7.9	7.9		
		25 ± 1		DO (mg/L)	8.3	8.3	8.3	8.3	8.5	8.5		
JLM/EH	9/16	24 Hr	1	pH	7.7	7.7	7.7	7.7	7.7	7.7		
		25 ± 1		DO (mg/L)	8.3	8.0	8.0	7.9	7.9	7.9		
		Renew	1	pH	8.1	8.0	8.0	7.9	7.9	7.8		
				DO (mg/L)	7.8	8.2	8.2	8.3	8.3	8.6		
JLM/GS	9/17	48 Hr	1	pH	7.8	7.8	7.8	7.8	7.8	7.8		
		25 ± 1		DO (mg/L)	7.7	7.7	7.7	7.6	7.6	7.5		
		Renew	1	pH	8.0	8.0	8.0	8.0	8.0	7.9		
				DO (mg/L)	7.5	7.6	7.6	7.7	7.7	8.1		
DT	9/18	72 Hr	1	pH	7.8	7.8	7.8	7.8	7.8	7.7		
		25 ± 1		DO (mg/L)	7.7	7.6	7.6	7.5	7.5	7.4		
		Renew	1	pH	8.0	8.1	8.1	8.0	8.0	8.0		
				DO (mg/L)	8.0	8.1	8.1	8.3	8.3	8.4		
EH	9/19	96 Hr	1	pH	7.5	7.6	7.6	7.6	7.6	7.6		
		25 ± 1		DO (mg/L)	8.3	8.0	8.0	7.9	7.9	7.9		
		Renew	1/2	pH	7.7	7.7	7.7	7.7	7.7	7.4		
				DO (mg/L)	7.9	8.0	8.0	8.1	8.1	8.5		
GS	9/20	120 Hr	1/2	pH	7.7	7.8	7.7	7.7	7.8	7.8		
		25 ± 1		DO (mg/L)	8.1	8.0	7.9	7.9	7.9	7.9		
		Renew	2	pH	8.0	8.0	7.8	7.8	7.7	7.7		
				DO (mg/L)	8.3	8.3	8.4	8.4	8.5	8.5		
DT/EH	9/21	144 Hr	2	pH	7.5	7.5	7.5	7.5	7.5	7.5		
		25 ± 1		DO (mg/L)	7.9	7.8	7.8	7.8	7.8	7.9		
		Renew	2	pH	7.7	7.6	7.7	7.6	7.6	7.6		
				DO (mg/L)	8.0	8.5	8.5	8.5	8.5	8.5		
GS	9/22	168 Hr	2	pH	7.8	7.7	7.7	7.7	7.7	7.7		
		25 ± 1		DO (mg/L)	7.8	7.7	7.6	7.6	7.6	7.6		

Appendix B

Ceriodaphnia dubia

BIO-AQUATIC TESTING, INC.

Carrollton, TX

REFERENCE TOXICANTS

Bio-Aquatic Testing conducts reference toxicant testing monthly for organisms cultured in-house. For studies requiring purchased organisms, reference toxicant testing is performed simultaneously. Reference toxicant testing validates data and measures organism consistency. Only reagent grade chemicals are used of the following choices: sodium laurel sulfate (SLS), copper sulfate, copper chloride, potassium chloride, and sodium chloride. Organism responses are tracked with control charts for each reference toxicant/organism combination. The data are examined for sensitivity trends and to determine if results are within EPA described limits.

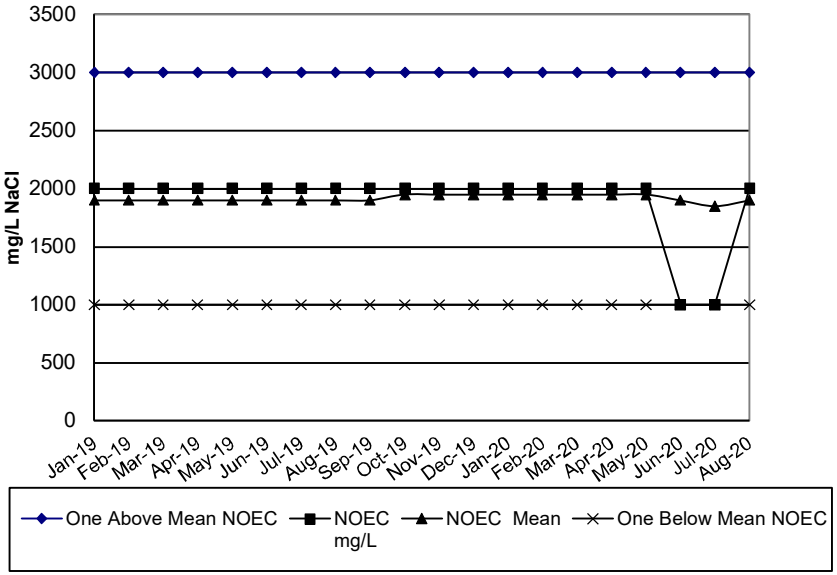
CHRONIC REFERENCE TOXICANT TEST RESULTS

DILUTION WATER:	Standard Synthetic Freshwater						
CHEMICAL:	Sodium Chloride						
DURATION:	3-Brood Chronic						
TEST NUMBER:	306						
PROJECT NUMBER:	77160						
START DATE:	8/25/2020						
START TIME:	13:36						
TOTAL NUMBER EXPOSED:	10 organisms per concentration						
CONCENTRATIONS (mg/L):	CON	250	500	1000	2000	3000	4000
NUMBER DEAD PER CONCENTRATION:	0	0	0	0	1	10	10
TEST METHODS:	As listed in EPA-821-R-02-013						
STATISTICAL METHODS:	SURVIVAL: Fisher's Exact Test REPRODUCTION: ANOVA-Dunnetts Test						

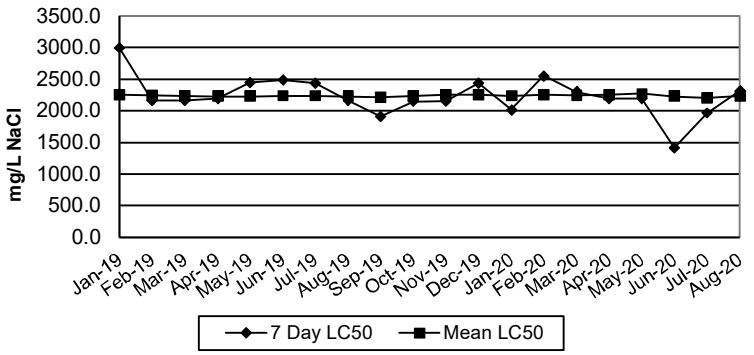
NOEC FOR SURVIVAL:	2000	mg/L
LOEC FOR SURVIVAL:	3000	mg/L
NOEC FOR REPRODUCTION:	500	mg/L
LOEC FOR REPRODUCTION:	1000	mg/L

PMSD: 12.3

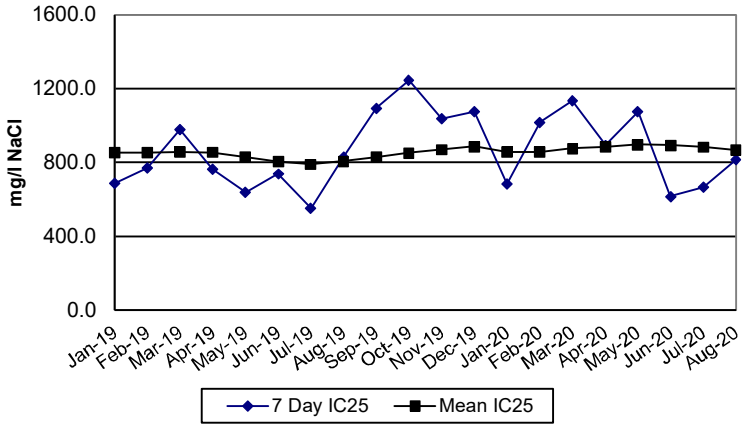
Ceriodaphnia Chronic Survival Control Chart



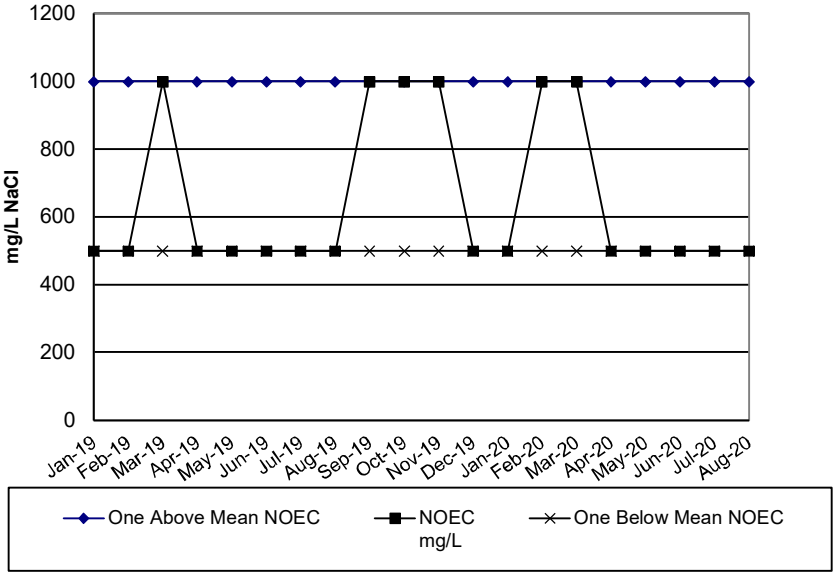
Ceriodaphnia 7-Day LC50



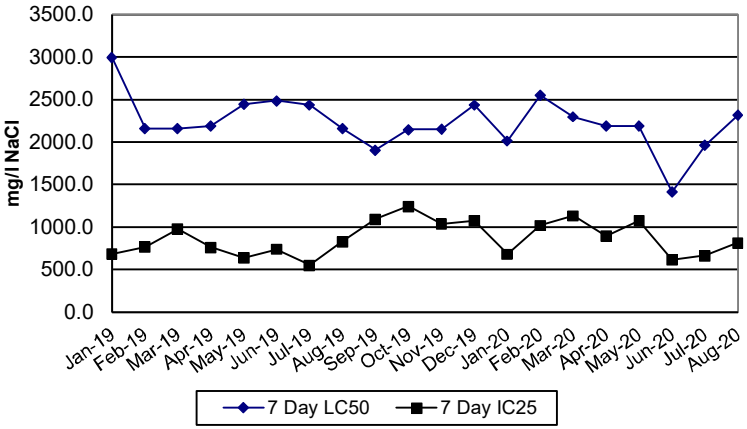
Ceriodaphnia 7-Day IC25



Ceriodaphnia Chronic Reproduction Control Chart



Ceriodaphnia 7-Day LC50 & IC25



Appendix B

Pimephales promelas

BIO-AQUATIC TESTING, INC.

Carrollton, TX

REFERENCE TOXICANTS

Bio-Aquatic Testing conducts reference toxicant testing monthly for organisms cultured in-house. For studies requiring purchased organisms, reference toxicant testing is performed simultaneously. Reference toxicant testing validates data and measures organism consistency. Only reagent grade chemicals are used of the following choices: sodium laurel sulfate (SLS), copper sulfate, copper chloride, potassium chloride, and sodium chloride. Organism responses are tracked with control charts for each reference toxicant/organism combination. The data are examined for sensitivity trends and to determine if results are within EPA described limits.

CHRONIC REFERENCE TOXICANT TEST RESULTS

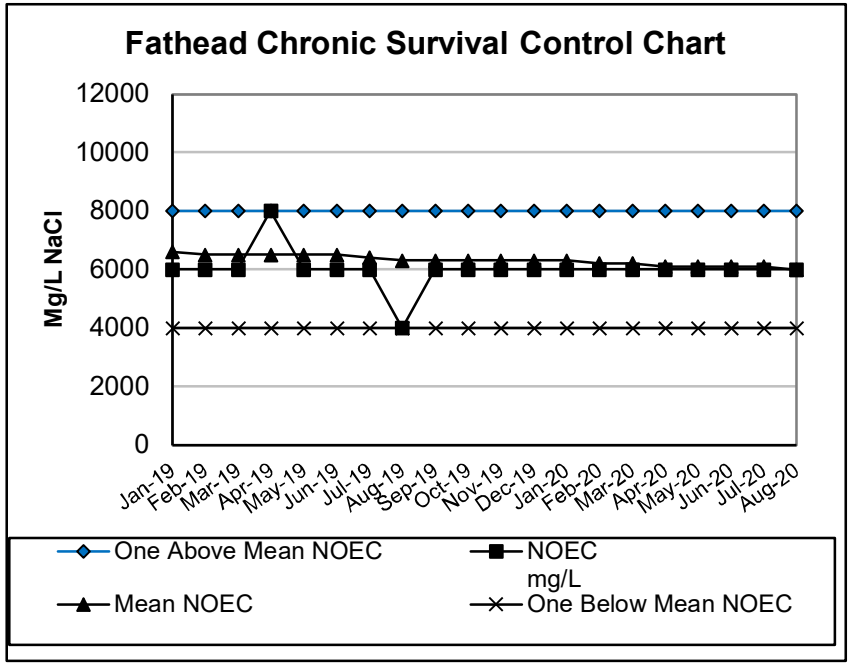
DILUTION WATER:	Standard Synthetic Freshwater						
CHEMICAL:	Sodium Chloride						
DURATION:	7 Days						
TEST NUMBER:	346						
PROJECT NUMBER:	77162						
START DATE:	8/27/2020						
START TIME:	14:47						
TOTAL NUMBER EXPOSED:	40 organisms per concentration						
CONCENTRATIONS (mg/L):	CON	2000	4000	6000	8000	10000	12000
NUMBER DEAD PER CONCENTRATION:	0	0	0	2	13	35	40

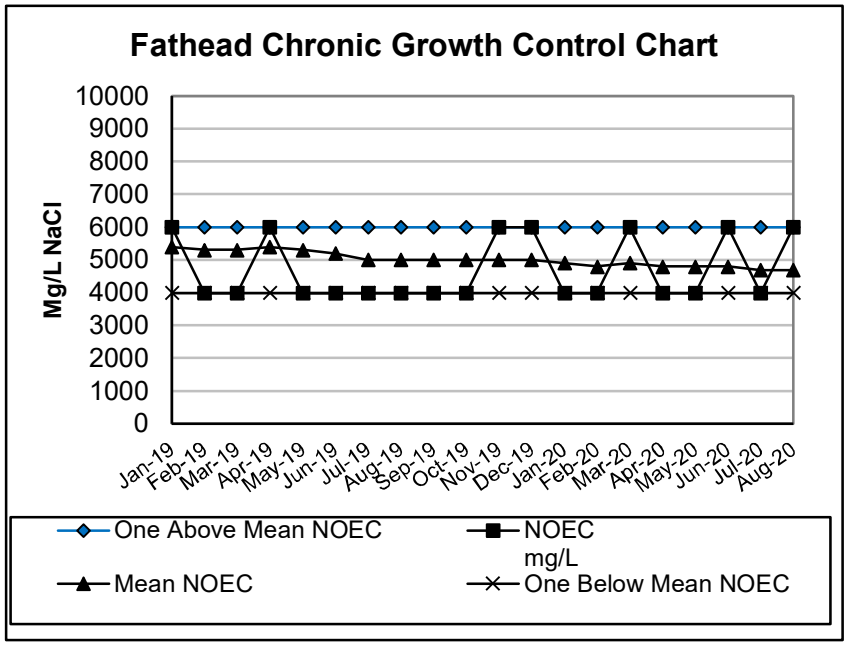
TEST METHODS: As listed in EPA-821-R-02-013

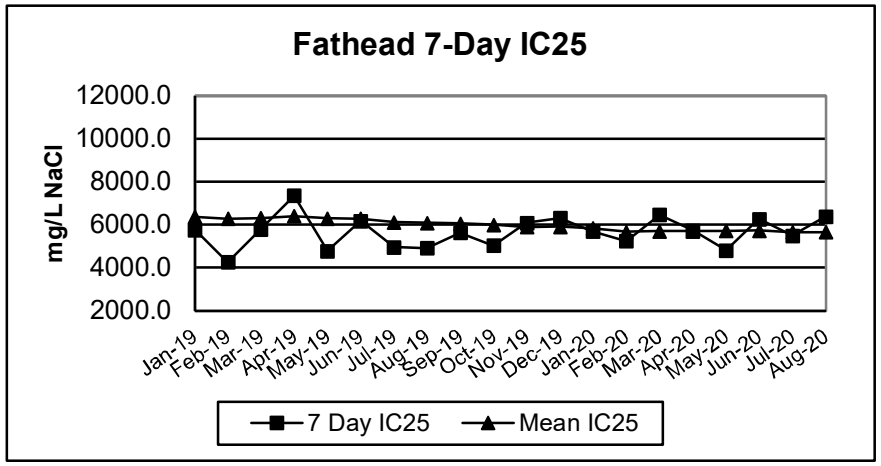
STATISTICAL METHODS: SURVIVAL: Steel's Many-One Rank Test
GROWTH: ANOVA-Dunnett's

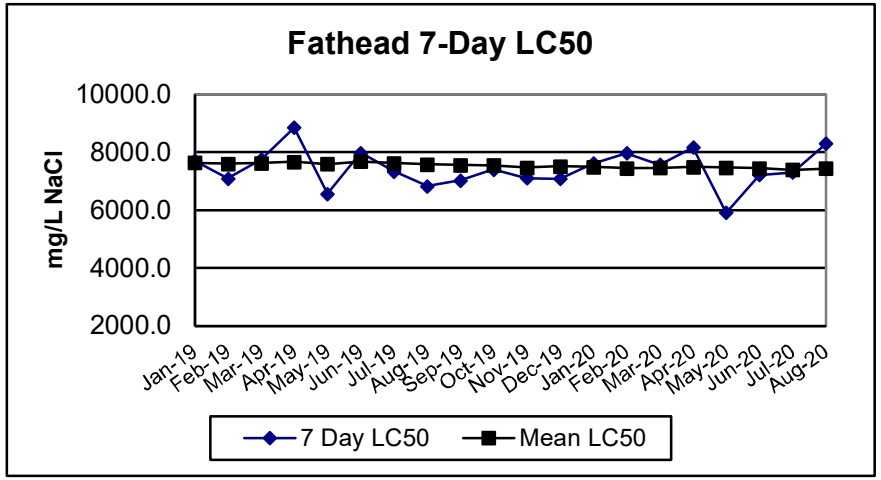
NOEC FOR SURVIVAL:	6000	mg/L
LOEC FOR SURVIVAL:	8000	mg/L
NOEC FOR GROWTH:	6000	mg/L
LOEC FOR GROWTH:	8000	mg/L

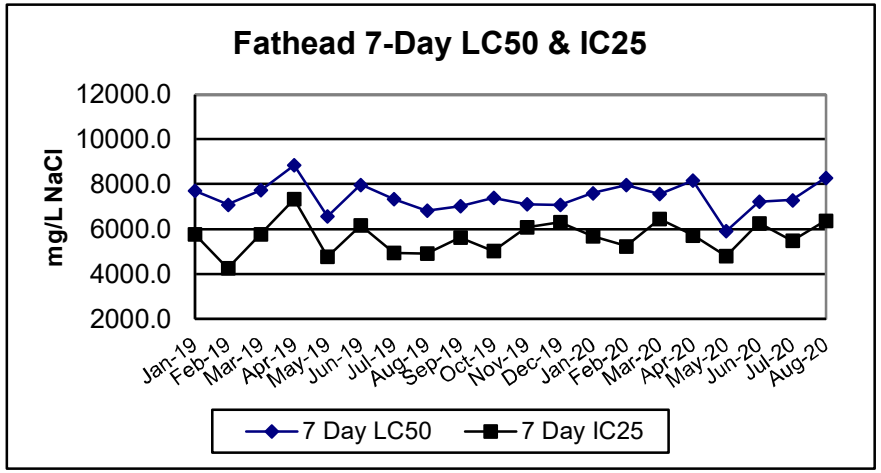
PMSD: 21.0


Fathead Chronic Survival Control Chart
Mg/L NaCl
12000
10000
8000
6000
4000
2000
0
Jan-19 Feb-19 Mar-19 Apr-19 May-19 Jun-19 Jul-19 Aug-19 Sep-19 Oct-19 Nov-19 Dec-19 Jan-20 Feb-20 Mar-20 Apr-20 May-20 Jun-20 Jul-20 Aug-20
One Above Mean NOEC
NOEC mg/L
Mean NOEC
One Below Mean NOEC


Fathead Chronic Growth Control Chart
Mg/L NaCl
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Jan-19 Feb-19 Mar-19 Apr-19 May-19 Jun-19 Jul-19 Aug-19 Sep-19 Oct-19 Nov-19 Dec-19 Jan-20 Feb-20 Mar-20 Apr-20 May-20 Jun-20 Jul-20 Aug-20
One Above Mean NOEC
NOEC mg/L
Mean NOEC
One Below Mean NOEC


Fathead 7-Day IC25
mg/L NaCl
12000.0
10000.0
8000.0
6000.0
4000.0
2000.0
Jan-19 Feb-19 Mar-19 Apr-19 May-19 Jun-19 Jul-19 Aug-19 Sep-19 Oct-19 Nov-19 Dec-19 Jan-20 Feb-20 Mar-20 Apr-20 May-20 Jun-20 Jul-20 Aug-20
7 Day IC25
Mean IC25


Fathead 7-Day LC50
mg/L NaCl
10000.0
8000.0
6000.0
4000.0
2000.0
Jan-19 Feb-19 Mar-19 Apr-19 May-19 Jun-19 Jul-19 Aug-19 Sep-19 Oct-19 Nov-19 Dec-19 Jan-20 Feb-20 Mar-20 Apr-20 May-20 Jun-20 Jul-20 Aug-20
7 Day LC50
Mean LC50


Fathead 7-Day LC50 & IC25
mg/L NaCl
12000.0
10000.0
8000.0
6000.0
4000.0
2000.0
Jan-19 Feb-19 Mar-19 Apr-19 May-19 Jun-19 Jul-19 Aug-19 Sep-19 Oct-19 Nov-19 Dec-19 Jan-20 Feb-20 Mar-20 Apr-20 May-20 Jun-20 Jul-20 Aug-20
7 Day LC50
7 Day IC25

APPENDIX C

LITERATURE REFERENCES

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U.S.E.P.A., 2002. Short-Term Methods For Estimating The Chronic Toxicity Of Effluents And Receiving Water To Freshwater Organisms (Fourth Edition) U.S. Environmental Protection Agency, Office of Water, Washington D.C., EPA-821-R-02-013.

U.S.E.P.A., 2012. Tropical Collector Urchin, *Tripneustes gratilla* (First Edition) U.S. Environmental Protection Agency, Office of Research and Development and Region 9, EPA-600-R-12-022.

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U.S.E.P.A., 1991. Technical Support Document For Water Quality-Based Toxics Control, U.S. Environmental Protection Agency, EPA-505-2-90-001.

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CHAIN-OF-CUSTODY SHEETS

Appendix D

BIO-AQUATIC TESTING, INC.
2501 MAYES RD., STE. 100
CARROLLTON, TX 75006
PH: 972-242-7750 FAX: 972-242-7749

CHAIN OF CUSTODY

☐ Bio Only: No Sample Left

Lab Id : 76521

Please Review & Complete Sections A, B, C, & D.

Sample No: 76521 – ____

Revision 2 Effective Date 8/25/2017

Check Sample No. : ✓ First, ____ Second, or ____ Third.

P.O. No:

Client: Nashville, City of
Facility: WPCP
Permit No: GA0039365
Outfall: 001
Client Contact: Brandon Rice
Client Phone: 2255396876

A. REVIEW SCHEDULED TEST(s):

Chronic	Ceriodaphnia dubia
Chronic	Pimephales promelas

Concentration: 12.5 25 50 69 100

To Ship the 1st Sample on: 9/7/2020

(For TX) Setup separate 24hr Acute Test? No

B. Use area below to make changes, if the Scheduled Test(s) in "A" are incorrect:

Freshwater Species					Saltwater Species	
C. dubia (water flea)	*D. pulex* (water flea)	*D. magna* (water flea)	*P. promelas* (minnow)	*Selenastrum* (green algae)	*M. beryllina* (minnow)	*Mysidopsis* (shrimp)
☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour

Notes: Annual Chronic Cerio/Fathead (AS)

C.

Sample ID or Location: (Outfall No. or Name)	Sample Type: E = Effluent RS = Rec. Stream S = Sediment	Sample Date From	Sample Date To	Sample Time (military) From	Sample Time (military) To	Grab or Composite	Sampled By: (Sign and Print Name)	Number Of Containers Shipped
1 001	E	9/13/20	9/14/20	930Am	800Am	C		1
2								
3								

D.

	Relinquished By:	Date	Time	Received By:	Date	Time
1		9/14/20	1030 Am		9-15-20	1330
2						
3						

Bio-Aquatic Sample Login

BAT sample personnel: ● Yes ○ No
Dechlorinate Sample: ○ Yes ◉ No
Dilution Water: ○ Receiving Stream ◉ Synthetic Lab

Date: 9-15	**Time:** 1330	**By:**	**Temperature:** 3.1 (C)	**IR#:** 002	
Chlorine: 0.1 mg/l	**Ammonia:** 0.25 mg/l	**Int. Sal\Cond:** 286 ppt/uS	**Adj. Salinity** ppt		
pH: 7.7	**Hardness:** 60 mg/l	**Other** Good			
DO: 9.1 mg/l	**Alkalinity:** 56 mg/l	**Condition:**			

BIO-AQUATIC TESTING, INC.
2501 MAYES RD., STE. 100
CARROLLTON, TX 75006
PH: 972-242-7750 FAX: 972-242-7749

CHAIN OF CUSTODY

☐ Bio Only: No Sample Left

Lab Id : 76521

Please Review & Complete Sections A, B, C, & D.

Sample No: 76521 -____

Revision 2 Effective Date 9/25/2017

Check Sample No. : ____ First, ____ Second, or ✓ Third.

P.O. No:

Client: Nashville, City of

Facility: WPCP

Permit No: GA0039365

Outfall: 001

Client Contact: Brandon Rice

Client Phone: 2295396976

A. REVIEW SCHEDULED TEST(s):

Chronic	Ceriodaphnia dubia
Chronic	Pimephales promelas

Concentration: 12.5 25 50 69 100

To Ship the 1st Sample on: 9/7/2020

(For TX) Setup separate 24hr Acute Test? No

B. Use area below to make changes, if the Scheduled Test(s) in "A" are incorrect:

Freshwater Species					Saltwater Species	
C. dubia (water flea)	*D. pulex* (water flea)	*D. magna* (water flea)	*P. promelas* (minnow)	*Selenastrum* (green algae)	*M. beryllina* (minnow)	*Mysidopsis* (shrimp)
☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour

Notes: Annual Chronic Cerio/Fathead (AS)

C.

Sample ID or Location: (Outfall No. or Name)	Sample Type: E = Effluent RS = Rec. Stream S = Sediment	Sample Date From	Sample Date To	Sample Time (military) From	Sample Time (military) To	Grab or Composite	Sampled By: (Sign and Print Name)	Number Of Containers Shipped
001	E	9/17/20	9/18/20	9AM	730AM	C	TBC Brandon Rice	1

D.

Relinquished By:	Date	Time	Received By:	Date	Time
TBC	9/18/20	830	Garrett Stephan	9/19/20	1133

Bio-Aquatic Sample Login

BAT sample personnel: ● Yes ○ No

Dechlorinate Sample: ○ Yes ◉ No

Dilution Water: ○ Receiving Stream ◉ Synthetic Lab

Date: 9/19/20 Time: 1133 By: CG Temperature: 4.5 (C) IR#: 002

Chlorine: <0.1 mg/l Ammonia: ~~20~~ ~~25~~ 0.25 mg/l Int. Sal\Cond: 209 ppt/uS Adj. Salinity ppt

pH: 7.1 Hardness: 69 mg/l LR Other

DO: 7.9 mg/l Alkalinity: 52 mg/l Condition: Good

Bio-Aquatic Lab ID: 76521

Page 35 of 41

Report Date: 10/09/2020 Revision 0

BIO-AQUATIC TESTING, INC
2501 MAYES RD., STE. 100
CARROLLTON, TX 75006
PH: 972-242-7750 FAX: 972-242-7749

CHAIN OF CUSTODY

☐ Bio Only: No Sample Left

Lab Id : 76521

Please Review & Complete Sections A, B, C, & D.

Sample No: 76521 -

Check Sample No. : ____ First, ✓ Second, or ____ Third.

P.O. No:

Client: Nashville, City of

Facility: WPCP

Permit No: GA0039365

Outfall: 001

Client Contact:

Client Phone:

A. REVIEW SCHEDULED TEST(s):

Chronic	Ceriodaphnia dubia
Chronic	Pimephales promelas

Concentration: 12.5 25 50 69 100

(For TX) Setup separate 24hr Acute Test? No

To Ship the 1st Sample on: 9/7/2020

B. Use area below to make changes, if the Scheduled Test(s) in "A" are incorrect:

Freshwater Species					Saltwater Species	
C. dubia (water flea)	*D. pulex* (water flea)	*D. magna* (water flea)	*P. promelas* (minnow)	*Selenastrum* (green algae)	*M. beryllina* (minnow)	*Mysidopsis* (shrimp)
☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour	☐Chronic ☐96 Hour ☐48 Hour ☐24 Hour

Notes: Annual Chronic Cerio/Fathead (AS)

C.

	Sample ID or Location: (Outfall No. or Name)	Sample Type: E = Effluent RS = Rec. Stream S = Sediment	Sample Date From	Sample Date To	Sample Time (military) From	Sample Time (military) To	Grab or Composite	Sampled By: (Sign and Print Name)	Number Of Containers Shipped
1	001	E	9/15/20	9/16/20	915	800	C	TBC	1
2									
3									

D.

	Relinquished By:	Date	Time	Received By:	Date	Time
1	TBC	9/16/20	900			
2						
3						

Bio-Aquatic Sample Login

Out of Hold time! 9-19-20 DT

BAT sample personnel: ● Yes ○ No

Dechlorinate Sample: ○ Yes ◉ No

Dilution Water: ○ Receiving Stream ◉ Synthetic Lab

Date:	Time:	By:	Temperature: (C)	IR#:			
Chlorine: mg/l	Ammonia: mg/l	Int. Sal\Cond: ppt/uS	Adj. Salinity ppt				
pH:	Hardness: mg/l	Other					
DO: mg/l	Alkalinity: mg/l	Condition:					

REGULATORY AGENCY TABLES

Appendix E

Table 1 (Sheet 1 of 4)

BIOMONITORING REPORT

Ceriodaphnia dubia SURVIVAL AND REPRODUCTION TEST

Permittee: Nashville, City of - WPCP

Permit No.: GA0039365

Outfall No.: 001

		Date/Time		Date/Time
Dates and times Composites were collected:	FROM:	9/13/2020 @ 09:30	TO:	9/14/2020 @ 08:00
	FROM:	9/17/2020 @ 09:00	TO:	9/18/2020 @ 07:30
	FROM:	@	TO:	@

Sample two was received out of hold time and was not used. The test was renewed with sample one until sample three was received. The test was then renewed and completed with sample three.

Test Initiation: Time: 14:20 Date: 9/15/2020

Dilution Water Used: [ ] Receiving Water [X] Synthetic Dilution Water

NUMBER OF YOUNG PRODUCED PER ADULT AT TEST TERMINATION

	EFFLUENT CONCENTRATION (%)					
REPLICATE	0%	12.5 %	25 %	50 %	69 %	100
A	24	24	30	25	28	27
B	25	26	30	27	25	23
C	28	23	22	23	25	28
D	27	31	27	23	21	31
E	27	25	24	21	25	25
F	19	33	32	19	19	32
G	19	26	25	24	25	34
H	26	21	28	27	29	22
I	24	25	24	24	20	22
J	25	D- 0	32	16	23	22
Surv. MEAN	24.4	26.0	27.4	22.9	24.0	26.6
Total MEAN	24.4	23.4	27.4	22.9	24.0	26.6
CV % [1]	12.8	14.5	13	15	13.6	17
PMSD	Acceptable Range 47 or Less					20.9 %

[1] Coefficient of Variation = (standard deviation/mean) x 100) Calculations are based on young of the surviving females. Males are designated (M), and dead females are designated (D) along with the number of neonates released prior to death. (E) anomalous value, spilled cup, or technician error.

Table 1 (Sheet 2 of 4)

BIOMONITORING REPORT

Ceriodaphnia dubia SURVIVAL AND REPRODUCTION TEST

Permittee: Nashville, City of - WPCP

Permit No.: GA0039365

Outfall No.: 001

PERCENT SURVIVAL

	EFFLUENT CONCENTRATION (%)					
TIme of Reading	0%	12.5 %	25 %	50 %	69 %	100 %
24 HOURS	100.0	100.0	100.0	100.0	100.0	100.0
48 HOURS	100.0	100.0	100.0	100.0	100.0	100.0
7-DAY	100.0	90.0	100.0	100.0	100.0	100.0

1. DUNNETT'S PROCEDURE OR STEEL'S MANY-ONE RANK TEST OR WILCOXON RANK SUM TEST
(with Bonferroni adjustment as appropriate for Sub-Lethality)
Is the mean number of young produced per adult significantly less (p=0.05) than the number of young per adult in the control for the % effluent corresponding to significant non-lethal effects?

CRITICAL DILUTION (69): _____YES _____X_____NO

If you report NO, enter a '0' on the DMR form for Parameter ***TWP3B****, other wise enter a '1'. This parameter is also referred to as the 7-DAY Ceriodaphnia Sub-Lethal Pass/Fail.*

2. FISHER'S EXACT TEST (as appropriate for Lethality)

Is the mean survival at test end significantly less (p=0.05) than the control's survival for the % effluent corresponding to lethality?

CRITICAL DILUTION (69): _____YES _____X_____NO

If you report NO, enter a '0' on the DMR form for Parameter ***TLP3B****, other wise enter a '1'. This parameter is also referred to as the 7-DAY Ceriodaphnia Lethal Pass/Fail.*

3. Enter the percent effluent corresponding to each NOEC/LOEC below:

a. NOEC Survival = 100 % Effluent (Parameter TOP3B)

b. LOEC Survival = Q* % Effluent (Parameter TXP3B)

c. NOEC Reproduction = 100 % Effluent (Parameter TPP3B)

d. LOEC Reproduction = Q* % Effluent (Parameter TYP3B)

Q* refers to a value that is not calculable

Table 1 (Sheet 3 of 4)
BIOMONITORING REPORT

Pimephales promelas SURVIVAL AND GROWTH TEST

Permittee: Nashville, City of - WPCP
Permit No.: GA0039365
Outfall No.: 001

		Date/Time		Date/Time
Dates and times Composites were collected:	FROM:	9/13/2020 @09:30	TO:	9/14/2020@ 08:00
	FROM:	9/17/2020 @09:00	TO:	9/18/2020@ 07:30
	FROM:	@	TO:	@

Sample two was received out of hold time and was not used. The test was renewed with sample one until sample three was received. The test was then renewed and completed with sample three.

Test Initiation: Time: 15:45 Date: 9/15/2020

Dilution Water Used: [ ] Receiving Water [X] Synthetic Dilution Water

DATA TABLE FOR GROWTH OF *Pimephales promelas*

Effluent Concentration	Average Dry Weight in milligrams (mg) per replicate					Mean Dry Weight (mg)	CV % [1]
	A	B	C	D	E		
0%	0.511	0.498	0.453	0.381	0.520	0.473	12.09
12.5 %	0.478	0.484	0.456	0.458	0.428	0.461	4.81
25 %	0.465	0.434	0.432	0.470	0.241	0.408	23.27
50 %	0.428	0.454	0.297	0.574	0.402	0.431	23.13
69 %	0.214	0.402	0.392	0.418	0.520	0.389	28.38
100 %	0.582	0.501	0.377	0.538	0.416	0.483	17.63
PMSD	Acceptable Range 30 or Less					26.5 %	

[1] Coefficient of Variation = (standard deviation/mean) x 100) ?= cannot be calculated due to 100% mortality or lab exception

DATA TABLE FOR SURVIVAL OF *Pimephales promelas*

Effluent Concentration	Percent Survival per replicate					Average % Survival			CV % [1]
	A	B	C	D	E	24 Hours	48 Hours	7-Day	
0%	87.5	100	100	100	100	100	97.5	97.5	5.73
12.5 %	100	100	100	87.5	100	100	100	97.5	5.73
25 %	75	100	100	100	37.5	100	100	82.5	33.20
50 %	100	87.5	62.5	100	100	100	100	90	18.11
69 %	37.5	75	62.5	87.5	100	100	100	72.5	33.16
100 %	87.5	100	62.5	100	75	100	97.5	85	19.17

Table 1 (Sheet 4 of 4)

BIOMONITORING REPORT

Pimephales promelas SURVIVAL AND GROWTH TEST

Permittee: Nashville, City of - WPCP

Permit No.: GA0039365

Outfall No.: 001

1. DUNNETT'S PROCEDURE OR STEEL'S MANY-ONE RANK TEST OR WILCOXON RANK SUM TEST (with Bonferroni adjustment as appropriate for Sub-Lethality)

Is the mean dry weight at 7 days significantly less (p=0.05) than the control's mean dry weight for the % effluent corresponding to significant non-lethal effects?

CRITICAL DILUTION (69) : _____YES ____X____NO

*If you report NO, enter a '0' on the DMR form for Parameter **TWP6C**, other wise enter a '1'. This parameter is also referred to as the 7-DAY Pimephales Sub-Lethal Pass/Fail.*

2. DUNNETT'S PROCEDURE OR STEEL'S MANY-ONE RANK TEST OR WILCOXON RANK SUM TEST (as appropriate for Lethality) Is the survival at 7 days significantly less (p=0.05) than the control's survival for % effluent corresponding to lethality?

CRITICAL DILUTION (69) : _____YES ____X____NO

*If you report NO, enter a '0' on the DMR form for Parameter **TLP6C**, other wise enter a '1'. This parameter is also referred to as the 7-DAY Pimephales Lethal Pass/Fail.*

3. Enter the percent effluent corresponding to each NOEC/LOEC below:

			For DMR Form:
a.	NOEC Survival =	100 % Effluent	(Parameter TOP6C)
b.	LOEC Survival =	Q* % Effluent	(Parameter TXP6C)
c.	NOEC Growth =	100 % Effluent	(Parameter TPP6C)
d.	LOEC Growth =	Q* % Effluent	(Parameter TYP6C)

Q* refers to a value that is not calculable