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Office of Water / Office of Wastewater Management/
Water Permits Division

Sampling Report for the Vessel General Permitting Program Pump Mortality Study

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SECTION 1
INTRODUCTION

EPA's vessel general permit requires several best management practices (BMPs) which are thought to reduce the numbers of living organisms discharged in ballast water. The objective of this bench-scale study is to explore the efficacy of one of those BMPs for Great Lakes bulk carriers (Lakers[1]) by determining if emptying ballast tanks by pumping creates greater mortality for larger organisms taken up and discharged in ballast water than emptying ballast tanks by gravity. This study took place September 8 to 10, 2014 at the Lake Superior National Estuarine Research Reserve (LSNERR) located in Superior, Wisconsin under the direction of the Office of Wastewater Management of the U.S. Environmental Protection Agency (EPA).

Samples were collected in accordance with procedures specified in the *Sampling and Analysis Plan for the Vessel General Permitting Program Pump Mortality Study* (SAP) and the *Quality Assurance Project Plan for Technical Support for the Vessel General Permitting Program – Pump Mortality Study* (QAPP). The SAP is provided in Appendix A of this report. Samples of fish and fish eggs for mortality analysis were collected by EPA's contractor Eastern Research Group, Inc. (ERG) and analyzed on-site by Great Lakes Environmental Center (GLEC) (subcontractor to ERG).

Section 2.0 of this report describes the mortality testing methodology and deviations from the SAP. Section 3.0 presents the analytical data collected during the sampling episode and ERG's evaluation of the data. Section 4.0 describes the quality assurance and quality control (QA/QC) procedures and results, and Section 5.0 presents references used in this document.

1.1 BACKGROUND

As the result of a 2006 court order, on February 6, 2009, EPA began permitting discharges incidental to the normal operation of vessels operating in a capacity as a means of transportation through the Vessel General Permit (VGP). The 2013 VGP (USEPA, 2013) includes general effluent limits applicable to all discharges; general effluent limits applicable to 27 specific discharge streams; narrative water-quality based effluent limits; inspection, monitoring, recordkeeping, and reporting requirements; and additional requirements applicable to certain vessel types. Ballast water is one of the applicable vessel discharges controlled under that permit.

During ballast water intake, a diverse community of live organisms present in both the water column and seafloor sediments is entrained into a vessel's ballast tanks (Ruiz and Reid, 2007). When Lakers ballast in a Great Lakes' port which has been colonized by an aquatic nuisance species (ANS), and then discharge their ballast in another Great Lakes' port, they have the potential to spread ANS within the Great Lakes (Rup et al., 2010; Briski et al., 2012). In Part 2.2.3.3 of the 2013 VGP, EPA included several permit conditions for ballast water management

[1] "Laker" is the common name for the large and uniquely designed and constructed dry bulk vessels (or carriers) used to transport bulk material commodities throughout the Great Lakes system. U. S. flag Lakers usually only transport goods on the four upper Great Lakes and connecting channels, as *most* are limited by their size from transiting the Welland Canal. The primary commodities transported by the Lakers include iron ore pellets, coal, grain, limestone, cement, sand, and salt.

for Lakers to reduce the likelihood of those vessels dispersing and spreading aquatic invasive species. One of those requirements is for vessels to use their ballast pumps to empty their ballast tanks, rather than gravity draining, to produce both shear and cavitational stresses on these organisms, theoretically resulting in higher mortality. Although pumping ballast water rather than gravity draining should result in additional organism mortality, EPA is aware of only one experimental study (USCG, 2013a) to support the BMP. This study examined only larval fish and did not investigate other life stages such as fish eggs that can also be drawn into ballast tanks. As such, EPA is actively gathering data on the mortality caused by pumps to other life-stages of fish.

1.2 Objectives and Scope

The objective of this bench-scale study was to determine if emptying ballast tanks by pumping creates greater mortality for fish eggs and fish (minnows) than emptying ballast tanks by gravity draining. Based on the results of the bench-scale testing, additional pilot or full scale testing may be conducted under future work assignments.

The general approach of this bench-scale study included the following steps:

- Collect fish eggs and small minnows from a laboratory-raised culture;
- Place the organisms into two feed tanks and a control tank;
- Gravity drain one feed tank into a collection net and count the number of live and dead organisms following gravity draining;
- Pump the second feed tank into a collection net and count the number of live and dead organisms following pumping. The pumping rate was adjusted to simulate the ballast pumping rate on a Laker;
- Determine the test handling mortality by analyzing live and dead organisms in the control tank;
- Using statistical analysis, determine the differences in mortality between the control, gravity draining and pumping for each organism type.

1.3 Location Selection

EPA conducted the bench-scale study at the LSNERR located in Superior, Wisconsin (see Figure 1-1). This facility was selected for the study due to its location on the shore of Lake Superior's St. Louis River estuary (a major shipping port) and the availability of Lake Superior estuary water for maintaining the organisms before and after testing. In addition, the facility provided laboratory and dock space needed for the test tanks and analytical equipment.

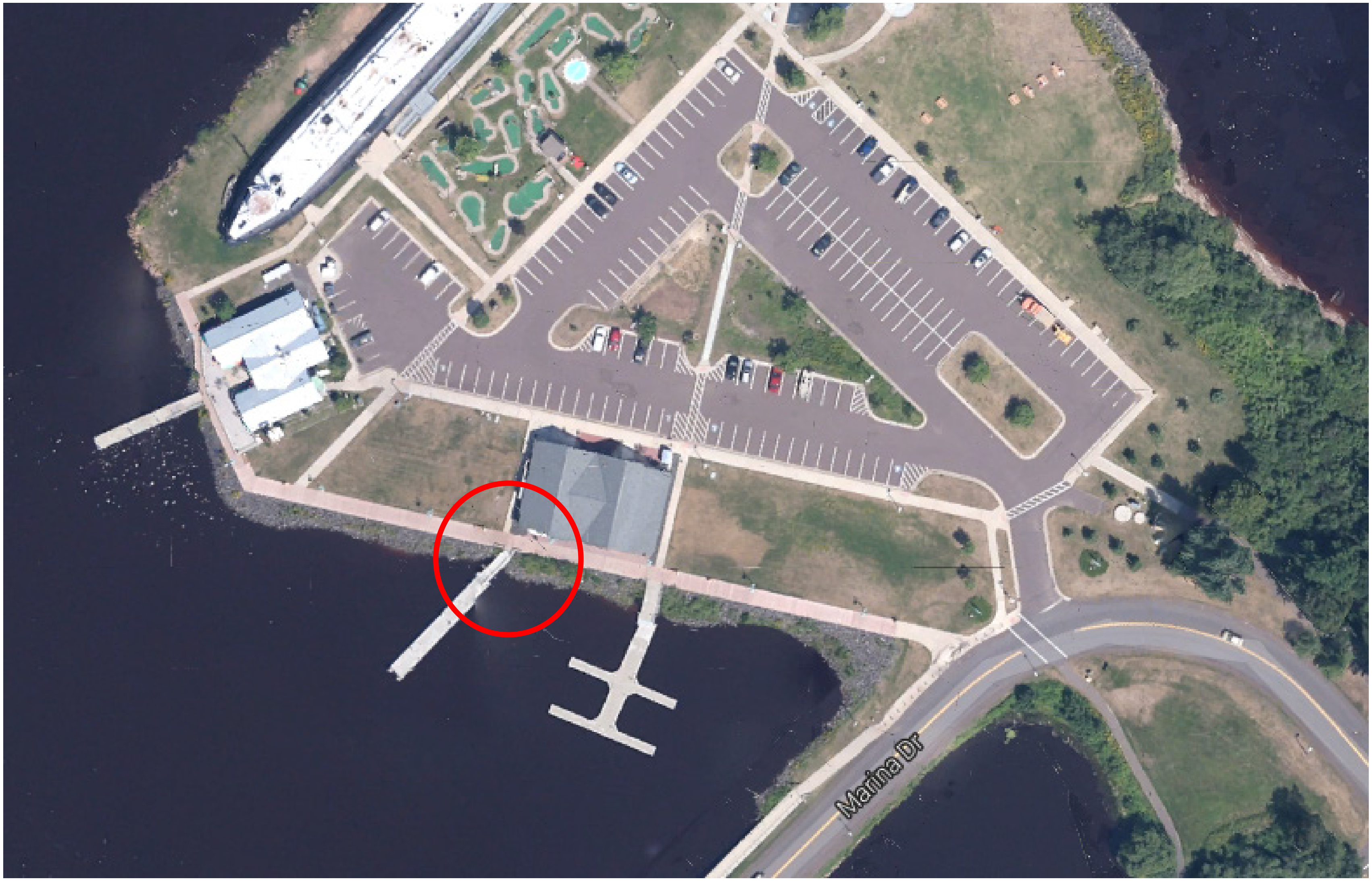


Figure 1-1. Diagram of the LSNERR with Red Circle Indicating the Dock Location where the Study Occurred

SECTION 2
MORTALITY TESTING

This section provides the detailed procedure that was used to conduct the bench-scale organism mortality tests at LSNERR. The bench-scale testing procedure was divided into three phases that included: (1) constructing the test apparatus including the tanks, piping, pumps, and post discharge organism collection nets; (2) obtaining small fish (native fathead minnows) and conducting the gravity drain, pump and control testing and live/dead sample analysis; and (3) obtaining fish eggs (native fathead minnow eggs) and conducting the gravity drain, pump and control testing and live/dead sample analysis. Testing for each organism group (fish eggs and minnows) was conducted separately. All tests had multiple replicates. The following subsections describe each of these three phases in greater detail.

Special certification requirements were required and obtained for this study to bring live fathead minnow eggs spawned at EPA's Mid-Continent Ecological Division (MED) laboratory in Duluth Minnesota to the LSNERR in Superior, Wisconsin test facility. Those included a Fish Health Certification (FHC) from a veterinarian for EPA's laboratory supplying the fathead minnow eggs and a Fish Import Permit from the Wisconsin Department of Agriculture to bring the eggs to Wisconsin. In addition, Fish Stocking Permits were required from the Wisconsin Department of Natural Resources for both the fathead minnow eggs and the fathead minnows obtained from a local minnow supplier. Copies of the MED FHC, the Fish Import Permit, and the Fish Stocking Permits are provided in Appendix B.

2.1 BENCH-SCALE TESTING APPARATUS

Figure 2-1, Figure 2-2, and Figure 2-3 are diagrams depicting the pump, gravity drain, and control system testing apparatus, respectively. Figure 2-4 is a site layout showing the arrangement of the test tanks and the sample receiving areas.

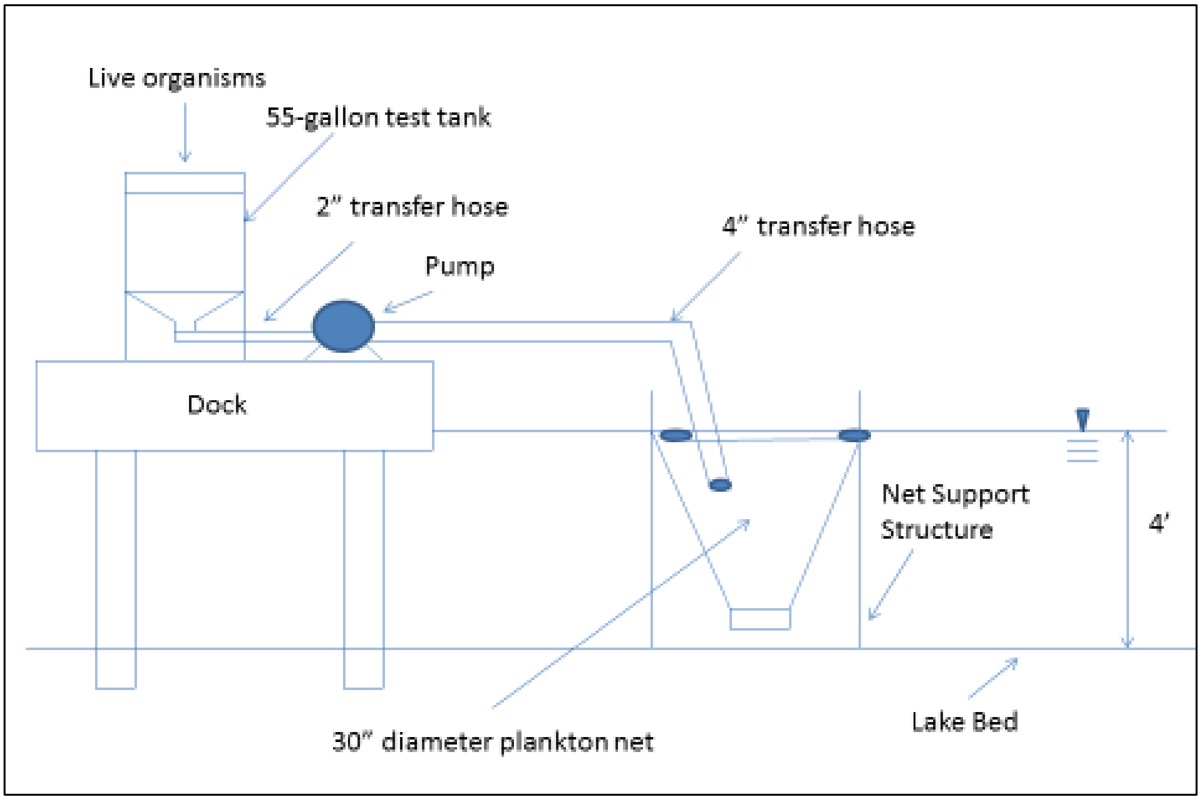


Figure 2-1. Pump System Testing Apparatus

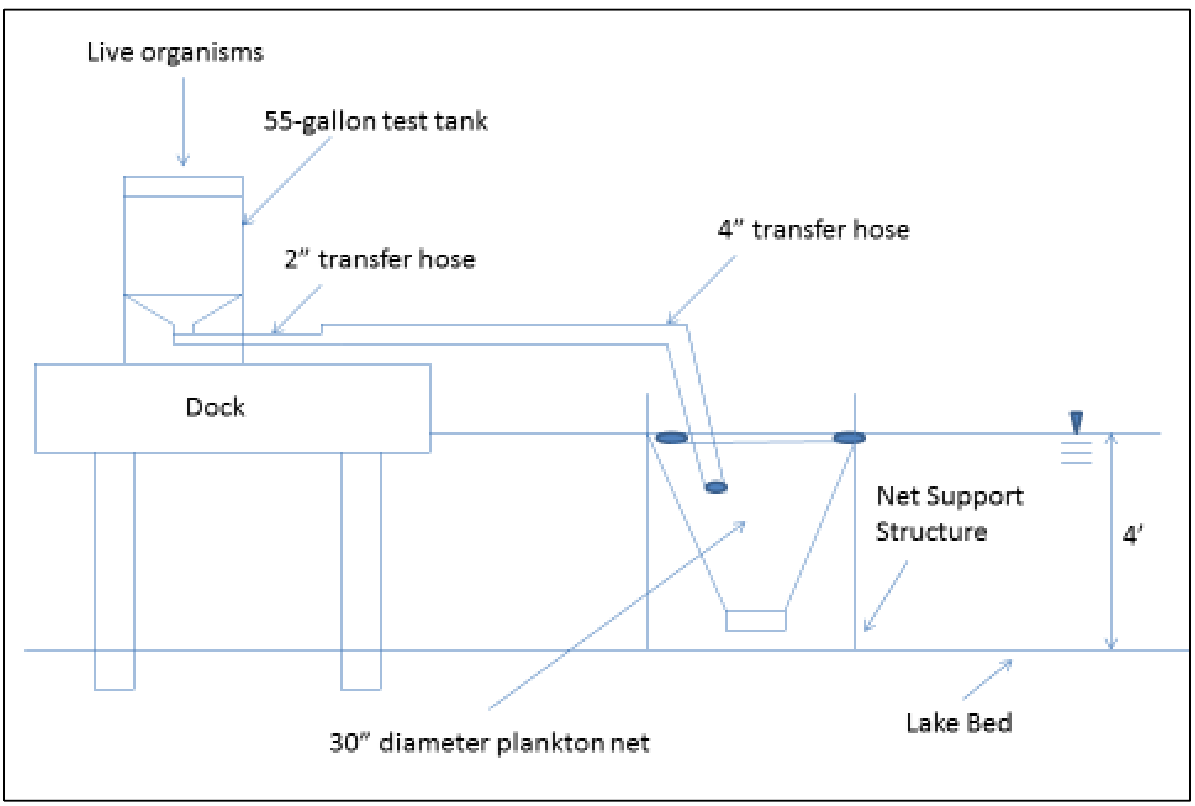


Figure 2-2. Gravity Drain System Testing Apparatus

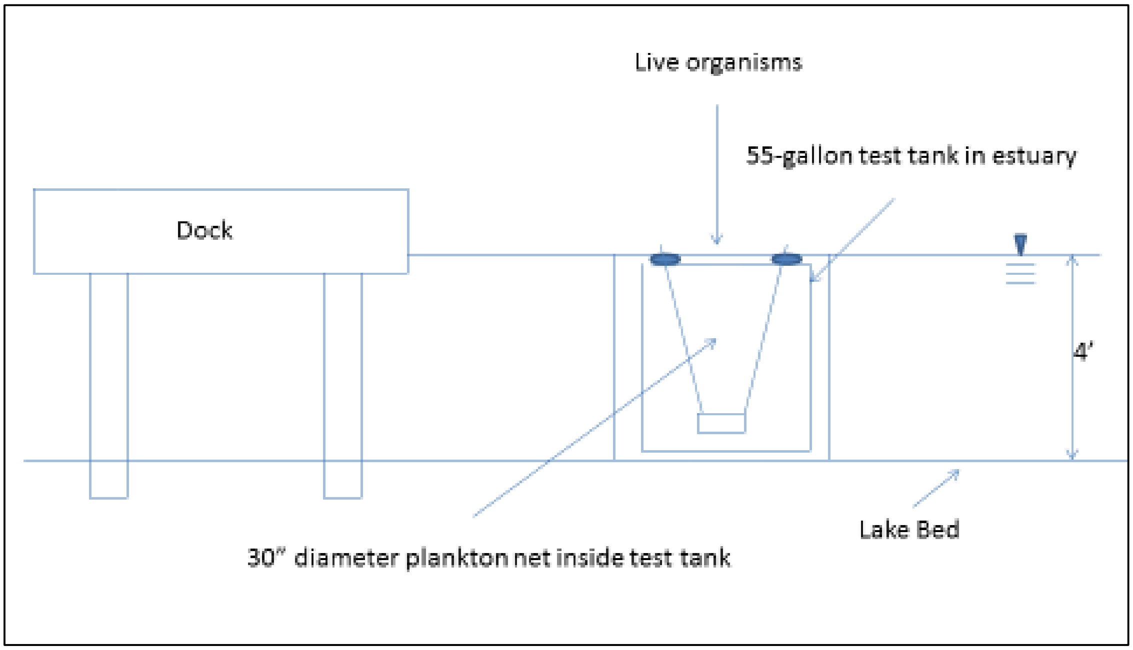


Figure 2-3. Control System Testing Apparatus

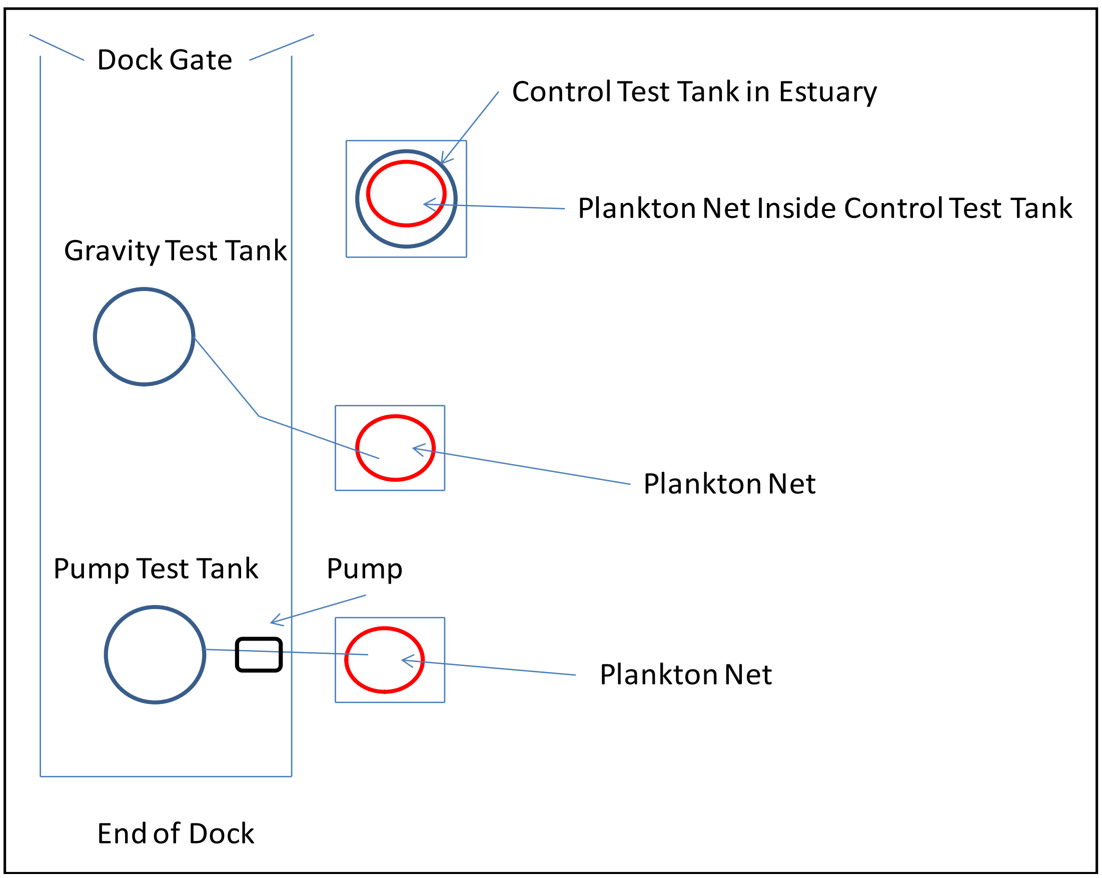


Figure 2-4. Layout of Testing System

The bench-scale testing system consisted of two 55-gallon open top plastic test tanks placed on concrete blocks (see Photographs 47, 52, and 53 in Appendix C). Each test tank had a diameter of 30 inches and a height of 35 inches. The pump and gravity drain tanks were fitted with a 2-inch diameter valve beneath the tanks for draining or pumping. The pump and gravity drain tanks were filled with water directly from the St. Louis River estuary using a pump. The pump and gravity drain tanks were directed into the plankton nets placed in approximately 4 feet of water in the estuary (see Photographs 2, 28, and 41 in Appendix C). The plankton nets had a 35 micron screen size (USEPA, 2010), were 30-inch diameter and were fitted with a 1-liter plastic bottle on the bottom to capture the organisms rinsed from the nets.

The pump tank discharged below the water line into the plankton net to simulate a ballast discharge through the sea chest. The gravity drain tank also discharged into the plankton net below the water line to simulate gravity draining by Lakers.[2] EPA placed the plankton nets in the estuary rather than in another receiving tank to buffer the force of the water and minimize the potential mortality that could be caused by the organisms contacting plankton nets during discharge. In addition, to further reduce mortality caused by organisms being forced into the plankton nets by water pressure, the pipe diameter on the pump tank discharge line was increased from 2 inches to 4 inches. This reduced the pressure and force with which test water was discharged into the plankton net to help counteract the force imposed by the pump. The gravity drain tank also included a pipe size increase from 2 inches to 4 inches near the middle of the pipe run in to provide consistency between the pump and gravity drain systems. To ensure against "contamination" of the test net with organisms in the water (and escape of live organisms inside the test nets), net supports were constructed to hold the nets approximately 8 inches above the water level. See Photographs 27, 28, and 51 in Appendix C.

To evaluate organism mortality caused by handling, the control tank (third tank) was submerged in the estuary adjacent to the pump and gravity drain collection nets. The control tank was lined with a 35-micron plankton net and suspended by the lifting winch. The control tank was submerged rather than drained to reduce possible organism mortality that could be caused by the change in elevation from the dock to the sample receiving area in the estuary. This net was also supported approximately 8 inches above the water level to guard against organism escape or entrainment of additional organisms from estuary water (see Photographs 7 and 28 in Appendix C).

To simulate ballast pump conditions from a Laker, EPA used a gasoline powered centrifugal trash pump with 2-inch diameter hose connected to the valve on the bottom of the pump tank (see Photograph 52 in Appendix C). The pump flow rate was calibrated by measuring the time needed to reduce the volume of a full 55 gallon tank to its overflow weir located at the tank bottom. Based on an average time of 35 seconds to empty the tank, EPA estimated the pump flow rate to be approximately 83 gallons per minute (gpm) at the hydrostatic head conditions observed during the study. For a 2-inch diameter flexible suction hose and a measured flow rate of 83 gpm, the flow rate per area of hose was calculated to be 26.4 gpm/in^2.

[2] According to Mr. Noel Bassett with The American Steamship Company and Mr. Jim Weakley at the Lake Carriers Association, American Lakers typically discharge ballast water through either their low or high sea chests below the water line.

According to ballast system design data for Lakers (USCG, 2013b), ballast water pumping rates range between 3,600 and 26,000 gpm resulting in a flow rate per area ranging between 31 and 46 gpm/in^2 depending on the diameter of the vessels ballast water piping. Other published information suggests that actual ballast water flow through the piping of Lakers is 10 ft/second and therefore ballast water treatment system testing should be conducted at flows ranging between 23 gpm and 40.1 gpm (Cangelosi et al., 2011). Although the flow per area for this study was less than the flow per area for Lakers (26.4 gpm/in^2), the flow rate (83 gpm) was above the flow rates suggested for testing (Cangelosi et al., 2011); therefore, the flows for this study appear appropriate for a bench-scale test to evaluate pump mortality.

2.2 Fish Mortality Testing

Approximately 1,500 fathead minnows were obtained from Hayward Bait in Hayward, Wisconsin, and were brought to the LSNERR in the early morning hours of September 9, 2014, and placed in 3 aerated 10-gallon aquaria established in the on-site laboratory (see Photographs 15, 19, and 20 in Appendix C). The fathead minnows ranged in size from approximately 1 inch in length to approximately 2.5 inches in length. Fathead minnows were selected for testing because they are native to Lake Superior and the St. Louis River. These minnows also tend to be relatively hardy, helping to ensure that there will be little handling and control mortality.

Mortality testing was conducted by first filling the pump and gravity drain tanks with approximately 50 gallons of St. Louis River estuary water and then adding approximately 100 fathead minnows collected from the stock aquaria. Once the minnows were added, the drain valves were opened and the minnows were allowed to flow by either gravity to a plankton net, or through the operating trash pump and into a plankton net. For the control tank submerged in the estuary, approximately 100 minnows were added directly into the net. Following a one hour recovery period, the nets were raised and the minnows were collected into 1-liter plastic sample bottles fastened to the bottom of the nets. The sample bottles were immediately removed from the nets following sample collection and the bottles transferred to the on-site laboratory where they were gently emptied into pre-labeled (control, pump and gravity) aerated aquaria containing St. Louis River estuary water (see Photographs 35 through 38 in Appendix C). Mortality assessment was conducted on-site by GLEC by removing subsamples of minnows from the appropriate aquaria and examining the minnows either under the microscope or with the naked eye to assess body or gill movement depending on fish size.

Table 2-1 shows the sample numbers and the times when the minnows were introduced into the nets and removed from the nets for each of the five replicate samples.

Table 2-1. Sample Numbers and Start and Stop Times for the Fathead Minnow Replicates

Replicate	Control		Gravity		Pump		Sample Numbers
	In	Out	In	Out	In	Out	
1	9:24	10:24	9:26	10:28	9:32	10:33	FC1, FG1, FP1
2	10:38	11:38	10:42	11:42	10:45	11:46	FC2, FG2, FP2
3	11:51	12:49	11:53	12:53	11:56	12:56	FC3, FG3, FP3
4	12:59	14:00	13:01	14:02	13:05	14:05	FC4, FG4, FP4
5	14:07	15:07	14:10	15:08	14:13	15:11	FC5, FG5, FP5

Throughout the testing period, the temperature, hardness and dissolved oxygen concentrations were monitored in the stock aquaria (aquaria 1 through 3) and the post discharge aquaria (aquaria 4 through 6) to ensure these conditions would not impart mortality on the minnows. Temperature data for each of the aquaria are provided in Table 2-2.

Table 2-2. Ambient and Aquarium Temperature Data for Fathead Minnow Testing

Time	Ambient (°C)[a]	Stock Aquaria 1 (°C)[b]	Stock Aquaria 2 (°C)[b]	Stock Aquaria 3 (°C)[b]	Control Aquaria 4 (°C)[b]	Pump Aquaria 5 (°C)[b]	Gravity Aquaria 6 (°C)[b]
8:45	19.5	20	20	19.5	20	20	19.5
9:50	19	20	20	20	20	20	19.5
10:50	19.5	20	20	20	19.5	19.5	20
12:00	19.5	20.5	20	20	20	21	20.5
13:07	20	20.5	20	20	20	20	20
14:16	21	NA	NA	NA	20.5	20	20

NA - All fish removed after last replicate so no data were collected.

[a] Ambient temperature of the St. Louis River estuary adjacent to the plankton nets receiving the minnows.

[b] Aquaria 1 through 3 hold stock minnows prior to testing, and aquaria 4 through 6 hold minnows after testing.

The hardness of the ambient water placed in Aquaria 1 through 5 was also measured and compared to the hardness of the water received from Hayward Bait (placed in Aquaria 6) to determine if hardness adjustments were necessary to prevent osmotic shock when the minnows were introduced to the St. Louis River estuary during testing. Hardness of the water from Hayward Bait in which the minnows were raised was determined to be 50 ppm (as $CaCO_3$) while the hardness of the ambient water in Aquaria 1 through 5 ranged from 90 ppm to 115 ppm as $CaCO_3$. Because of the difference in hardness between the water received from Hayward Bait and the ambient water in Aquaria 1 through 5, the hardness of the Hayward Bait water (Aquaria 6) was adjusted to 100 ppm (as $CaCO_3$) by slowly adding commercially purchased mineral water before transferring minnows to Aquaria 1 to 3 to prevent osmotic shock. Hardness data collected during the entire testing period are provided in Table 2-3.

Table 2-3. Ambient and Aquarium Hardness Data for Fathead Minnow Testing

Time	Ambient (ppm)[a]	Stock Aquaria 1 (ppm)[b]	Stock Aquaria 2 (ppm)[b]	Stock Aquaria 3 (ppm)[b]	Control Aquaria 4 (ppm)[b]	Pump Aquaria 5 (ppm)[b]	Gravity Aquaria 6 (ppm)[b]
9:00	130	110	90	110	115	115	50[c]
11:00	100	140	100	100	115	110	100
13:34	120	120	110	110	120	120	100

[a] Ambient hardness of the St. Louis River estuary adjacent to the plankton nets receiving the minnows.

[b] Aquaria 1 through 3 hold stock minnows prior to testing, and aquaria 3 through 6 hold minnows after testing.

[c] Aquaria 6 was used as the original stock tank that all minnows were placed in after receipt from Hayward Bait. Water from Hayward Bait was used to fill Aquaria 6 resulting in a stock tank hardness of 50 ppm. Hardness was slowly adjusted in Aquaria 6 to approximately 100 ppm before transferring minnows to Aquaria 1 through 3 to prevent osmotic shock.

The dissolved oxygen of the water in each aquaria was also measured along with the dissolved oxygen of the St. Louis River estuary (ambient) to verify sufficient dissolved oxygen was available to sustain the minnows. Dissolved oxygen data, measured by a direct reading probe, for the estuary and the aquariums are provided in Table 2-4. The data show that dissolved oxygen was present in the stock aquaria and the ambient water.

Table 2-4. Ambient and Aquarium Dissolved Oxygen Data for Fathead Minnow Testing

Time	Ambient (mg/L)[a]	Stock Aquaria 1 (mg/L)[b]	Stock Aquaria 2 (mg/L)[b]	Stock Aquaria 3 (mg/L)[b]	Control Aquaria 4 (mg/L)[b]	Pump Aquaria 5 (mg/L)[b]	Gravity Aquaria 6 (mg/L)[b]
8:45	9.3	5.0	3.1	5.5	NA[c]	NA[c]	NA[c]
9:50	8.6	6.0	3.8	6.4	NA[c]	NA[c]	NA[c]
10:50	9.0	4.2	3.9	5.5	8.7	8.2	8.6
12:00	9.1	5.2	4.9	6.6	9.0	8.0	9.0
13:08	8.7	5.0	4.8	7.1	9.1	9.0	9.1
14:18	9.8	NA[c]	NA[c]	NA[c]	9.8	9.6	9.4

[a] Ambient dissolved oxygen of the St. Louis River estuary adjacent to the plankton nets receiving the minnows.

[b] Aquaria 1 through 3 hold stock minnows prior to testing, and aquaria 3 through 6 hold minnows after testing.

[c] Aquaria did not contain minnows and therefore dissolved oxygen was not measured.

2.3 FISH EGG MORTALITY TESTING

Live fathead minnow eggs were obtained from the MED laboratory in Duluth on September 10, 2014 for testing at the LSNERR. The eggs, hatched and raised by MED, were 3 days old, had a diameter of 1 millimeter, and included eyes and heartbeats that could be viewed under the microscope to assess living/dead. According to researchers at MED, the 3-day old eggs were within hours of hatching into larvae and were therefore most susceptible to environmental stress. Fathead minnow eggs were selected for testing because they are native to Lake Superior and the St. Louis River. See Photographs 50 and 55 in Appendix C.

For each test, the pump, gravity drain and control tanks were filled with approximately 50 gallons of St. Louis River estuary water and dosed with approximately 250 fathead minnow eggs. Due to the number of available eggs, EPA conducted only three replicate tests with the eggs (compared to five for fish). For each test, the eggs were introduced first into the control tank, then to the gravity drain tank and finally to the pump tank. Dosing and discharge of the three tanks occurred within a 15 minutes period for each replicate. For the gravity drain and pump tanks, the tank drain valves were opened and discharged to the receiving nets within 1 minute of dosing the eggs into the tanks. Once the eggs entered the plankton nets suspended in the estuary, the one hour recovery period began. Following the one-hour recovery period, the plankton nets were raised and rinsed using a hand sprayer to wash the eggs into the 1-liter receiving bottled fastened to the bottom of the nets. Once the nets were completely raised and rinsed, the 1-liter receiving bottle was immediately taken into the laboratory for mortality assessment under the dissecting microscopes (see Photographs 43 through 46 in Appendix C).

Table 2-5 shows the sample numbers and the times when the eggs were introduced into the nets and removed from the nets for each of the three replicate samples.

Table 2-5. Fathead Minnow Egg Mortality Testing Start and Stop Times and Corresponding Sample Numbers

Replicate	Control		Gravity		Pump		Sample Numbers
	In	Out	In	Out	In	Out	
1	9:44	10:44	9:58	10:59	9:51	10:52	EC1, EG1, EP1
2	11:05	12:02	11:13	12:12	11:07	12:07	EC2, EG2, EP2
3	12:15	13:13	12:20	13:18	12:17	13:16	EC3, EG3, EP3

Prior to testing, the temperature of the water in which the eggs were received (Stock Bottle 1) was measured and compared to the temperature of the St. Louis River estuary to determine if acclimation was required. As shown in Table 2-6, the temperature of the estuary was 17.5°C, and the temperature of Stock Bottle 1 was 20°C prior to the start of testing. Since the temperature difference between the water in the estuary and the water holding the eggs was less than 5°C, no further temperature acclimation was required. Temperature data measured for the St. Louis Rivers estuary and in the stock and sample bottles throughout testing are provided in Table 2-6.

Table 2-6. Fathead Minnow Egg Temperature Data Throughout the Testing Period

Time	Ambient °C[a]	Stock Bottle 1 °C[a]	Control Bottle 2 °C[a]	Gravity Bottle 3 °C[a]	Pump Bottle 4 °C[a]
9:25	17.5	20	NA[b]	NA[b]	NA[b]
10:15	17.5	19	17	17	17
11:18	17	20	16	16.5	16.5
13:15	16.5	NA[b]	16.5	16.5	16

[a] Bottle 1 contains stock fathead minnow eggs from MED prior to testing, and bottles 2 through 4 hold fathead minnow eggs following testing.

[b] NA - no eggs in bottle so measurement was not obtained.

Water hardness was also measured using an aquarium hardness test kit and compared to the hardness of the estuary to determine whether the eggs would need to be acclimated to prevent osmotic shock upon entering the receiving nets. Hardness of the estuary was measured at 90 ppm (as $CaCO_3$), and the hardness of the water provided with the fathead minnow eggs was 100 ppm (as $CaCO_3$). Since the hardness of the estuary and the hardness of the water in which the eggs were raised were within 10 percent, no hardness adjustments were made. Hardness data measured during testing are provided in Table 2-7.

Table 2-7. Ambient and Fathead Minnow Egg Container Hardness Data

Time	Ambient (ppm)	Stock Bottle 1 (ppm)[a]	Control Bottle 2 (ppm)[a]	Gravity Bottle 3 (ppm)[a]	Pump Bottle 4 (ppm)[a]
9:25	90	100	NA[b]	NA[b]	NA[b]
11:20	100	110	90	90	90

[a] Bottle 1 contains stock fathead minnow eggs from MED prior to testing, and bottles 2 through 4 hold fathead minnow eggs following testing.

[b] NA - no eggs in bottle so measurement was not obtained.

The dissolved oxygen of the water in each bottle was also measured along with the dissolved oxygen of the St. Louis River estuary (ambient) to verify sufficient dissolved oxygen was available to sustain the eggs. Dissolved oxygen data, measured by a direct reading probe, for the estuary and the bottles containing the eggs are provided in Table 2-8. The data show that sufficient dissolved oxygen was present in the ambient water, the stock bottle containing the stock of eggs prior to testing, and in the sample bottles of eggs after testing.

Table 2-8. Ambient and Fathead Minnow Egg Container Dissolved Oxygen Data

Time	Ambient (mg/L)	Stock Bottle 1 (mg/L)[a]	Control Bottle 2 (mg/L)[a]	Gravity Bottle 3 (mg/L)[a]	Pump Bottle 4 (mg/L)[a]
9:25	9.8	7.8	NA[b]	NA[b]	NA[b]
10:15	10.3	8.2	9.1	10.1	9.7
11:20	9.3	7.3	10.4	10.6	9.4
13:15	9.9	NA[b]	10.4	10.1	9.8

[a] Bottle 1 contains stock fathead minnow eggs from MED prior to testing, and bottles 2 through 4 hold fathead minnow eggs following testing.

[b] NA - no eggs in bottle so measurement not obtained.

2.4 QUALITY ASSURANCE/QUALITY CONTROL

Analytical quality control was measured by assessing overall data completeness, by comparing mortality counts of consecutive subsamples (precision), and by comparing duplicate mortality counts of the same subsample performed by two different analysts (accuracy). These data are discussed in Section 4.1. Field quality control was evaluated by assessing the integrity of the stock organisms and by measuring the variability among replicates for the control, gravity drain, and pump tests. These results are discussed in Section 4.2.

2.5 DEVIATIONS FROM THE SAMPLING AND ANALYSIS PLAN

The study proceeded as specified in the SAP with the deviations described in Table 2-9.

Table 2-9. Deviations from the Sampling and Analysis Plan

Deviation	Description
Pump Flow Rate	ERG intended to operate the pump flow rate at 32 gpm to simulate the testing protocols used by the Great Ships Initiative (Cangelosi et al., 2011); however, the minimum flow that could be obtained from the gasoline driven trash pump used for the study was 83 gpm (26.4 gpm/in^2) at the head pressure provided from the test tank. Although the flow rate from the trash pump used for this study is significantly greater than that of pump flow rate used for testing in the Great Ships Initiative protocols, the flow rate is slightly below the calculated unit area design maximum flow rate (gpm/in^2 of pipe) found on Lakers pumping ballast water, which ranges from 31 gpm/in^2 to 46 gpm/in^2 (USCG, 2013b). Since the pump flow rate used for this study (83 gpm, 26.4 gpm/in^2) is above the flow rate used by the Great Ships Initiative (32 gpm) but below the expected unit area flow rate for Lakers (31 to 46 gpm/in^2), the results should provide a reasonable estimate of the mortality caused by pumping and gravity draining ballast water for the two organism types.

Table 2-9. Deviations from the Sampling and Analysis Plan

Deviation	Description
Fathead Minnow Egg Test Replicates	ERG had intended to conduct 4 replicates using the 2,000 fathead minnow eggs provided by the MED laboratory; however, only 3 replicate tests could be completed since more eggs were required per test than originally planned. The SAP estimated that 3 eggs per gallon would be sufficient for estimating mortality; however, after further evaluation, the sampling team decided to use approximately 5 eggs per gallon to guarantee sufficient egg recovery in the plankton nets for mortality analysis.

SECTION 3
RESULTS AND DISCUSSION

This section presents the data collected during the pump mortality study. Mortality results for fathead minnows and fathead minnow eggs are presented in Section 3.1. Section 3.2 provides a summary of the data, including graphic representations along with a discussion regarding how the data may be used to evaluate the impact of pumping ballast water rather than gravity draining on the mortality of small fish and fish eggs. All raw mortality data provided by GLEC are provided in Appendix D of this report.

3.1 LABORATORY AND FIELD ANALYTICAL RESULTS

Mortality results for the fathead minnows and the fathead minnow eggs are provided in Table 3-1 and Table 3-2, respectively. The two tables present the live/dead counts for each tank (control, gravity drain, and pump) and for each replicate. The estimated percent mortality for each replicate and test tank is also included.

Table 3-1. Fathead Minnow Mortality Data for the Control, Gravity Drain and Pump Tanks

Replicate	Condition	Control Tank	Gravity Drain Tank	Pump Tank
1	Dead Minnows	2	0	65
	Live Minnows	115	113	56
	Total Minnows	117	113	121
	Percent Mortality	**1.7%**	**0.0%**	**53.7%**
2	Dead Minnows	0	0	51
	Live Minnows	152	121	67
	Total Recovered Minnows	152	121	118
	Percent Mortality	**0.0%**	**0.0%**	**43.2%**
3	Dead Minnows	0	1	31
	Live Minnows	93	94	58
	Total Recovered Minnows	93	95	89
	Percent Mortality	**0.0%**	**1.1%**	**34.8%**
4	Dead Minnows	0	0	45
	Live Minnows	154	127	51
	Total Recovered Minnows	154	127	96
	Percent Mortality	**0.0%**	**0.0%**	**46.9%**
5	Dead Minnows	0	0	72
	Live Minnows	139	195	86
	Total Recovered Minnows	139	195	158
	Percent Mortality	**0.0%**	**0.0%**	**45.6%**

Table 3-2. Fathead Minnow Egg Mortality Data for the Control, Gravity Drain and Pump Tanks

Replicate	Condition	Control Tank	Gravity Drain Tank	Pump Tank
1	Dead Eggs	2	1	1
	Live Eggs	127	26	124
	Total Recovered Eggs	129	27	125
	Percent Mortality	**1.6%**	**3.7%**	**0.8%**
2	Dead Eggs	0	0	2
	Live Eggs	40	25	55
	Total Recovered Eggs	40	25	57
	Percent Mortality	**0.0%**	**0.0%**	**3.5%**
3	Dead Eggs	0	1	0
	Live Eggs	1	56	38
	Total Recovered Eggs	1	57	38
	Percent Mortality	**0.0%**	**1.8%**	**0.0%**

3.2 DATA ANALYSIS AND DISCUSSION

To analyze the mortality data for the minnows and eggs, EPA took the weighted average mortality rate across the replicates (weighted by sample size of recovered organisms) and calculated the 95% confidence intervals around these point estimates (see Table 3-3). EPA also plotted the weighted average percent mortalities and confidence intervals for fathead minnows and fathead minnow eggs for each of the test tanks (see Figure 3-1 and Figure 3-2, respectively). (Note that negative mortality rate is not plausible, so the minimum lower bound is 0%.)

Table 3-3. Weighted Average Percent Mortality for the Control, Gravity Drain and Pump Test Tanks

Organism	Weighted Average Percent Mortality and 95% Confidence Interval		
	Control Tank[a]	**Gravity Drain Tank[a]**	**Pump Tank[a]**
Fathead Minnows	0.3% ± 0.4%	0.2% ± 0.3%	45.4% ± 3.4%
Fathead Minnow Eggs	1.2% ± 1.6%	1.9% ± 2.5%	1.4% ± 2.1%

[a] Average calculated from five replicates for fathead minnows and three replicates for fathead minnow eggs; weighted by the number of fish in each tank.

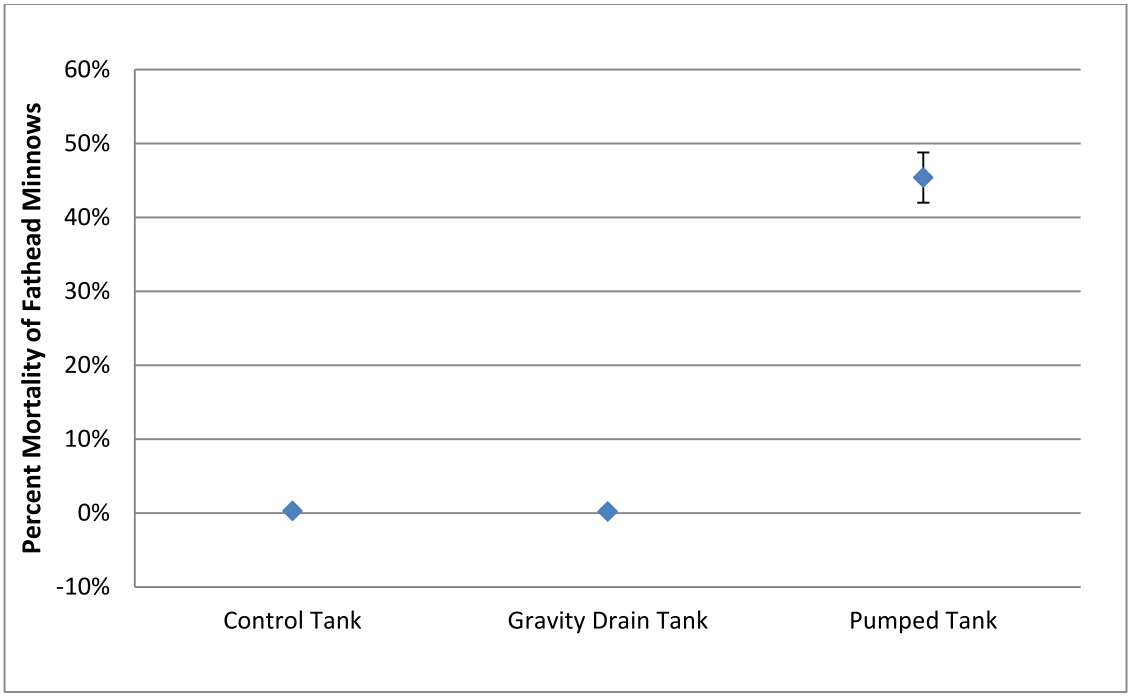
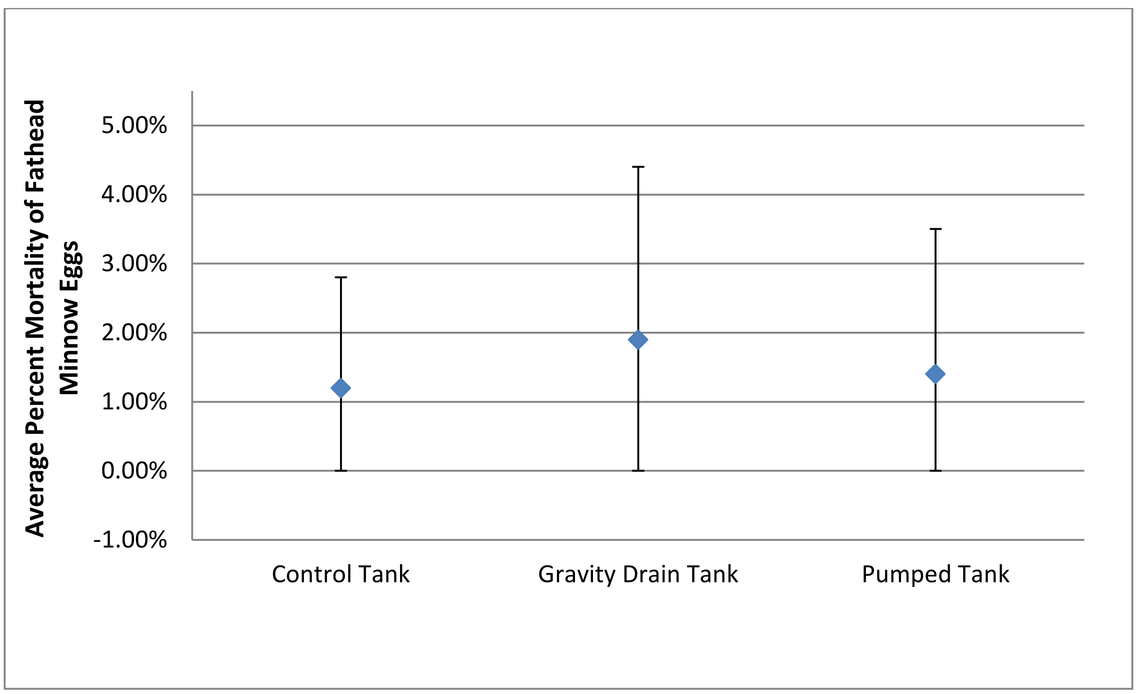


Figure 3-1. Weighted Average Percent Mortality and 95% Confidence Interval of Fathead Minnows



Note that negative mortality rate is not plausible, so the minimum lower bound is 0%.

Figure 3-2. Weighted Average Percent Mortality and 95% Confidence Interval of Fathead Minnow Eggs

To evaluate the impact of pumping ballast water rather than gravity draining on the mortality of small fish and fish eggs, EPA compared the weighted average percent mortality and variability in the control against the weighted average percent mortality and variability of the pump and gravity drain tests. Variability for each tank type and organism was measured using the 95 percent confidence interval around the average; this generates the upper and lower bounds. The average and upper bound control mortality were then compared to the average and lower bound pump and gravity drain mortalities to determine if the pump and gravity drain tests caused more mortality than the control. EPA also used the Wilcoxin signed-rank test, a non-parametric statistical hypothesis test, to assess whether mortality was significantly greater in the pump tank than in the gravity drain and control tanks (Wilcoxin, 1945).

<u>Fathead Minnows</u>

As indicated in Table 3-3 and depicted graphically in Figure 3-1, the upper bound of the 95 percent confidence interval on percent mortality for the fathead minnow control tank is 0.7% (mean of 0.3% plus 0.4%) and the lower bound mortality rate for the gravity drain tank and pump tank are 0% (0.2 minus 0.3%) and 41.9% (45.4% minus 3.4%), respectively. (Note that negative mortality rate is not plausible, so the minimum lower bound is 0%). For the gravity drain tank, the lower bound percent mortality is below the upper bound for the control tank mortality, verifying there is no difference in mortality between the control and gravity drain tank (i.e., gravity draining does not cause mortality). However, for the pump tank, the lower bound percent mortality is much larger than the upper bound for the control tank (41.9% versus 0.7%) verifying that pumping does impact mortality of fathead minnows. The Wilcoxin signed-rank test also found that fathead minnow mortality was significantly greater in the pump treatment than in both the gravity ($p<0.0001$) and control treatments ($p<0.0001$). Rates of fish mortality between control and gravity drain treatments were not significantly different.

One explanation as to why some minnows survived pumping while others were killed may be linked to their introduction in to the impeller cavity of the pump head. Observations made of the minnows discharged from the pump show that the minnows that died before reaching the laboratory were completely dismembered or were swimming erratically after discharge. Minnows that survived 1 hour after passing through the pump appeared healthy immediately after passing through the pump. These observations suggest that if minnows enter the pump's impeller cavity at the point where the impeller is passing the intake port, then they will likely be injured or killed. If the minnows enter the impeller cavity between the impeller blades, then they could be swept through the impeller cavity unharmed without contacting either the impeller or the cavity walls, or experiencing excessive shear or cavitational stress.

The mortality data obtained from this study using adult fathead minnows are only partially comparable to the study conducted by the U.S. Coast Guard (USCG) using Asian carp larvae on the Illinois River in 2011 (USCG, 2013a). First, published literature indicates that mortality of fish larvae is higher than fry or adult fish in many marine or freshwater species (Dahberg, 1979). Secondly, fathead minnows are a different species than Asian carp and possibly less susceptible to the shear stress caused by centrifugal pumps.

<u>Fathead Minnow Eggs</u>

As indicated in Table 3-3 and depicted graphically in Figure 3-2, the upper bound of the 95 percent confidence interval on percent mortality for the fathead minnow egg control tank is

2.8% (mean of 1.2% plus 1.6%) and the lower bound mortality rate for the gravity drain tank and pump tank are 0% (1.9% minus 2.5%) and 0% (1.4% minus 2.1%), respectively. (Note that negative mortality rate is not plausible, so the minimum lower bound is 0%.) Since the lower bound for the gravity drain and pump tanks are both below the upper bound for the control, there is no statistically significant difference in mortality between the control tank and the gravity drain or pump tanks, verifying that neither gravity draining nor pumping increases the mortality of fathead minnow eggs. The Wilcoxin signed-rank test also found that fathead minnow egg mortality was not significantly different between the pump, gravity drain and control tanks.

A likely explanation as to why fish eggs survived gravity draining and pumping is that their small size (approximately 1 millimeter diameter) allows them to enter the impeller cavity of the pump without significant contact with the blades or the impeller cavity walls or experiencing excessive shear or cavitational stress.

<u>*Conclusion*</u>

Based on this bench-scale study, it appears that ballast water pumps have measurable mortality for larger organisms such as fish, but that mortality is not measurable for fish eggs. Although the data from this study indicate roughly one-half of adult minnows and essentially no minnow eggs will be killed when passing through a centrifugal pump at unit flow rates comparable to the ballast water flows on a Laker, these data are not definitive of ballast water pump mortality due, in part, to differences in pump design. For example, ballast pump data provided by Phil Moore, Interlake Steamship Company (Moore, 2014) show that main ballast pumps on some of their vessels have 30 inch diameter impellers with a 30^{o} blade angle, a 2.5 inch gap between the outer edge of the impeller and the cavity walls, and operate at speeds of 690 revolutions per minute (RPM). Note that the ballast pump gap is larger than the length of the largest fish used for this testing, and much larger than the diameter of the fish eggs. In contrast, the impeller diameter on the trash pump used for this study was 5 inches and had a 0.008 to 0.014 inch (0.20 to 0.36 mm) gap between the outer edge of the impeller and the cavity walls.[3] The angle of the blades and the speed of the impeller used for this study were unknown. Note also that the trash pump gap is much smaller than the length/width of the fish used for this testing and is somewhat smaller than the diameter of the fish eggs. Because of these significant differences in physical size of the pumps, EPA can make only generalized conclusions based on the mortality caused by the centrifugal pump used in this study and the mortality that would be expected by main ballast pumps on Lakers. Future research evaluating ballast pump mortality could involve a similar study conducted on-board a Laker using actual main ballast pumps. Such a study would also account for other significant differences in physical size and configuration between our simulation and actual ballast water systems that could affect organism mortality, such as vessel ballast water piping and tankage.

[3] Wacker-Neuson 2” diameter centrifugal trash pump powered by a 4.8 horsepower Honda gasoline engine.

SECTION 4
DATA QUALITY

Quality assurance/quality control (QA/QC) procedures applicable to this sampling episode are outlined in the QAPP for this program, approved by EPA on May 16, 2014, and its amendment dated August 29, 2014. This section describes the quality control practices used to assess the precision and accuracy of the analytical data presented in Section 3.0. Quality control (QC) practices used for this sampling episode include the duplicate mortality counts and laboratory quality control checks.

4.1 ANALYTICAL QUALITY CONTROL

ERG verified that laboratory performance was acceptable by verifying that all samples received by the laboratory were analyzed within the method-specific holding times and that the quality checks of the mortality data, as specified by the QAPP, were conducted. Data review biologists from GLEC prepared a written data review narrative (see Appendix E) describing any qualifications of the data. The laboratory quality control measures for mortality analysis of fathead minnows and fathead minnow eggs are described below.

4.1.1 Completeness

Completeness is defined in terms of the percentage of data that were collected and deemed to be acceptable for use in this study. The goals for this study were a minimum of 80% sampling completeness and 90% analytical completeness resulting in a minimum overall completeness of 72% (determined by multiplying sampling and analytical completeness goals).

For the fathead minnow tests, all 15 of the targeted samples were collected and analyzed resulting in a sampling and analytical completeness of 100%. For the fathead minnow eggs, ERG had originally planned to complete 4 replicates, but due to a lack of eggs, only 3 replicates were completed, resulting in sampling completeness of 75%. All 9 fathead minnow egg samples from the 3 replicates were analyzed and the data deemed acceptable resulting in an analytical completeness of 100%. The overall completeness for the study was calculated to be 89% (24 of 27 samples collected, and 24 of 24 samples analyzed).

4.1.2 Precision

Precision is a measure of the agreement among repeated measurements and is quantitatively assessed by calculating the relative percent difference (RPD) between duplicate sample results. For this study, precision was measured by comparing the counts of living/dead organisms for every 10th subsample[4] to counts from the subsequent subsample (i.e., every 11th subsample). Per the requirements of the QAPP, duplicate recounts were conducted at a frequency

[4] For minnows, a subsample consisted of collecting a fraction of the total number of minnows in each sample and analyzing the sample for live/dead organisms. The number of subsamples collected from the minnows samples received in the laboratory ranged between 3 and 10. For fathead minnow eggs, a subsample consisted of a small aliquot (20 to 30 milliliters) of water obtained from the primary sample bottle and analyzed for live/dead organisms. The number of subsamples collected from the egg samples received in the laboratory ranged between 7 and 13.

of 10%, and the RPD between the duplicate counts samples was calculated to determine if the target RPD of ± 10 % was achieved. Table 4-1 and Table 4-2 show the results of the duplicate mortality counts and the calculated RPDs for minnows and eggs, respectively.

For dead minnows, only 1 of the 33 duplicate quality assurance subsamples exceeded the target RPD of 10%. For live minnows, only 3 of the 33 duplicate quality assurance subsamples exceeded the target RPD of 10%. The elevated RPD of 66.7% for one live minnow subsample is a result of the low number of minnows in the sample. For dead fathead minnow eggs, only 1 of the 10 duplicate quality assurance subsamples exceeded the target RPD, and for live fathead minnow eggs, none of the quality assurance subsamples exceeded the target RPD.

Table 4-1. Duplicate Sample Results and Calculated RPDs for Fathead Minnows

Treatment Type	Treatment Replicate	Sub-Sample Number	Original Sample Result (Dead)	Duplicate Sample Result (Dead)	RPD	Original Sample Result (Alive)	Duplicate Sample Result (Alive)	RPD
Control	1	2	1	1	0.0%	8	8	0.0%
Control	1	3	0	0	NC	7	7	0.0%
Control	1	4	0	0	NC	9	9	0.0%
Gravity	1	1	0	0	NC	14	14	0.0%
Gravity	1	6	0	0	NC	6	6	0.0%
Pump	1	2	11	12	8.7%	2	2	0.0%
Pump	1	2	8	8	0.0%	3	3	0.0%
Pump	1	2	12	13	8.0%	1	1	0.0%
Control	2	2	0	0	NC	8	8	0.0%
Control	2	4	0	0	NC	11	11	0.0%
Gravity	2	1	0	0	NC	13	13	0.0%
Pump	2	1	13	17	26.7%	12	15	22.2%
Pump	2	3	13	14	7.4%	5	4	22.2%
Pump	2	2	7	7	0.0%	4	4	0.0%
Control	3	1	0	0	NC	15	15	0.0%
Control	3	1	0	0	NC	17	17	0.0%
Gravity	3	1	0	0	NC	23	22	4.4%
Pump	3	1	2	2	0.0%	9	9	0.0%
Pump	3	1	4	4	0.0%	11	11	0.0%
Control	4	1	0	0	NC	14	14	0.0%
Gravity	4	2	0	0	NC	12	11	8.7%
Control	4	4	0	0	NC	14	14	0.0%
Pump	4	1	9	9	0.0%	1	1	0.0%
Pump	4	1	10	11	9.5%	16	17	6.1%
Gravity	4	12	0	0	NC	8	8	0.0%
Pump	4	3	2	2	0.0%	9	9	0.0%
Control	5	3	0	0	NC	12	12	0.0%
Control	5	3	0	0	NC	11	11	0.0%

Table 4-1. Duplicate Sample Results and Calculated RPDs for Fathead Minnows

Treatment Type	Treatment Replicate	Sub-Sample Number	Original Sample Result (Dead)	Duplicate Sample Result (Dead)	RPD	Original Sample Result (Alive)	Duplicate Sample Result (Alive)	RPD
Gravity	5	3	0	0	NC	9	9	0.0%
Pump	5	1	4	4	0.0%	9	9	0.0%
Pump	5	1	14	13	7.4%	1	2	66.7%
Pump	5	6	3	3	0.0%	12	12	0.0%
Gravity	5	16	0	0	NC	11	11	0.0%

NC = Not calculated.

Table 4-2. Duplicate Sample Results and Calculated RPDs for Fathead Minnow Eggs

Treatment Type	Treatment Replicate	Sub-Sample Number	Original Sample Result (Dead)	Duplicate Sample Result (Dead)	RPD	Original Sample Result (Alive)	Duplicate Sample Result (Alive)	RPD
Control	1	1	0	0	NC	19	19	0.0%
Gravity	1	1	0	0	NC	4	4	0.0%
Gravity	1	3	0	0	NC	7	7	0.0%
Pump	1	4	1	1	0.0%	32	32	0.0%
Control	2	3	0	0	NC	8	8	0.0%
Gravity	2	3	0	0	NC	6	6	0.0%
Pump	2	3	2	1	66.7%	15	15	0.0%
Control	2	12	0	0	NC	1	1	0.0%
Pump	3	1	0	0	NC	16	16	0.0%
Gravity	3	3	0	0	NC	12	12	0.0%

NC = Not calculated.

4.1.3 Accuracy

Accuracy is a measure of the agreement between a measured value and a reference of "true" value. In this study, the values in question are whether or not each organism type is alive or dead. Live organisms will exhibit either movement (with or without a stimulus) for small fish, or a heartbeat and movement within the egg for fish eggs. Possible accuracy errors are movements created by water currents that make a dead organism appear alive and non-responsive organisms that appear dead but are actually alive. To determine the accuracy of the live or dead analysis, a second GLEC analyst observed every 10th subsample and independently assessed mortality. This accuracy check was conducted by the second analyst immediately following counting by the first analyst to guard against organism death due to prolonged exposure to microscope lights. Accuracy is then measured by duplicate live/dead counts of the same subsample. The target difference between the duplicate counts of the same subsample should be less than 10 percent, resulting in 90 percent accuracy.

Accuracy data are provided in Table 4-3 and Table 4-4 for fathead minnows and fathead minnow eggs, respectively. For fathead minnows, only 1 of the 33 quality assurance subsamples for live and dead analysis had accuracy less than 90 percent. For fathead minnow eggs, only one of the 10 quality assurance subsamples had accuracy less than 90% and this was likely caused by the very low number of organisms in the sample.

Table 4-3. Accuracy Data for Fathead Minnow Quality Assurance Subsamples

Treatment Type	Treatment Replicate	Sub-Sample Number	Original Sample Result (Dead)	Duplicate Sample Result (Dead)	Dead Organism Accuracy	Original Sample Result (Alive)	Duplicate Sample Result (Alive)	Live Organism Accuracy
Control	1	2	1	1	100%	8	8	100%
Control	1	3	0	0	100%	7	7	100%
Control	1	4	0	0	100%	9	9	100%
Gravity	1	1	0	0	100%	14	14	100%
Gravity	1	6	0	0	100%	6	6	100%
Pump	1	2	11	12	92%	2	2	100%
Pump	1	2	8	8	100%	3	3	100%
Pump	1	2	12	13	92%	1	1	100%
Control	2	2	0	0	100%	8	8	100%
Control	2	4	0	0	100%	11	11	100%
Gravity	2	1	0	0	100%	13	13	100%
Pump	2	1	13	17	76%	12	15	80%
Pump	2	3	13	14	93%	5	4	80%
Pump	2	2	7	7	100%	4	4	100%
Control	3	1	0	0	100%	15	15	100%
Control	3	1	0	0	100%	17	17	100%
Gravity	3	1	0	0	100%	23	22	96%
Pump	3	1	2	2	100%	9	9	100%
Pump	3	1	4	4	100%	11	11	100%
Control	4	1	0	0	100%	14	14	100%
Gravity	4	2	0	0	100%	12	11	109%
Control	4	4	0	0	100%	14	14	100%
Pump	4	1	9	9	100%	1	1	100%
Pump	4	1	10	11	91%	16	17	94%
Gravity	4	12	0	0	100%	8	8	100%
Pump	4	3	2	2	100%	9	9	100%
Control	5	3	0	0	100%	12	12	100%
Control	5	3	0	0	100%	11	11	100%
Gravity	5	3	0	0	100%	9	9	100%
Pump	5	1	4	4	100%	9	9	100%
Pump	5	1	14	13	108%	1	2	50%
Pump	5	6	3	3	100%	12	12	100%
Gravity	5	16	0	0	100%	11	11	100%

Table 4-4. Accuracy Data for Fathead Minnow Eggs Quality Assurance Subsamples

Treatment Type	Treatment Replicate	Sub-Sample Number	Original Sample Result (Dead)	Duplicate Sample Result (Dead)	Dead Organism Accuracy	Original Sample Result (Alive)	Duplicate Sample Result (Alive)	Live Organism Accuracy
Control	1	1	0	0	100%	19	19	100%
Gravity	1	1	0	0	100%	4	4	100%
Gravity	1	3	0	0	100%	7	7	100%
Pump	1	4	1	1	100%	32	32	100%
Control	2	3	0	0	100%	8	8	100%
Gravity	2	3	0	0	100%	6	6	100%
Pump	2	3	2	1	50%	15	15	100%
Control	2	12	0	0	100%	1	1	100%
Pump	3	1	0	0	100%	16	16	100%
Gravity	3	3	0	0	100%	12	12	100%

4.2 FIELD QUALITY CONTROL

Field quality control was evaluated by first verifying that the organisms used in the study were alive prior to testing and then evaluating the variability between replicate tests. The following subsections describe each of these field quality controls.

4.2.1 Integrity of Stock Organisms

One indicator of field quality control was verification that the fathead minnows and fathead minnow eggs were alive prior to testing. To verify the fathead minnows were alive prior to each test replicate, the physical condition of the minnows was observed and any that were floating or appeared dead were removed. To verify the eggs were alive prior to testing, GLEC collected 10 subsamples of eggs from the stock and analyzed the eggs under the microscope. The data, provided in Table 4-5, indicate that in 8 of the 10 subsamples, all the eggs were alive prior to testing. Only two subsamples had any eggs that were dead. These results verify that the fathead minnow eggs received from MED were alive prior to testing.

Table 4-5. Mortality Analysis Results for Fathead Minnow Eggs Prior to Testing

Sample	Live	Dead	Indeterminate	% Mortality
1	5	0	2	0.0%
2	7	0	1	0.0%
3	7	4	1	36.4%
4	7	0	0	0.0%
5	14	0	1	0.0%
6	18	1	2	5.3%
7	8	0	1	0.0%
8	13	0	0	0.0%

Table 4-5. Mortality Analysis Results for Fathead Minnow Eggs Prior to Testing

Sample	Live	Dead	Indeterminate	% Mortality
9	9	0	1	0.0%
10	5	0	2	0.0%

4.2.2 Variability

Another field quality control measure is the variability among replicate tests for each organism and tank type (pump, gravity drain, and control). For fathead minnows, the average was estimated for each of the 5 replicates in the control tank; then an average of these 5 averages was taken and the 95 percent confidence interval around this average was estimated. The same procedure was conducted for the gravity drain and pump tanks. Similarly, for fathead minnow eggs, an average was estimated for each of the 3 replicates and then an average of these 3 averages was taken and the 95 percent confidence interval calculated.

As indicated in Table 4-6, the lower bound of the 95 percent confidence interval on percent mortality for the fathead minnow pump tank is 38.0% (44.8% minus 6.8%) and the upper bound mortality rate is 55.6% (44.8% plus 6.8%). Four of the five replicates are within this range, which indicates that the methodology is fairly replicable and consistent. The 95 percent confidence interval on percent mortality for the fathead minnow control tank is 0% (0.3% minus 0.8%) through 1.1% (0.3% plus 0.8%). Replicate 1 has a mean of 1.7%, which is outside this confidence interval. This implies there is some variability across replicates for the control tank. The other four point estimates are all zero and thus fall within the confidence interval. The 95 percent confidence interval for the gravity tank is 0 to 0.7% (0.2% minus and plus 0.5%). Once again the four zero values fall within this range but the average for the replicate where a single fish died is outside this range. These two samples lying outside the confidence intervals do not necessarily invalidate the results; when evaluating the incidence of rare events (i.e., low probability events, such as a fish dying in the control tank) the traditional measures of deviation often do not perform well. For instance, these intervals both include negative values which are not feasible outcomes (there cannot be negative dead fish), and thus the lower bounds are reported as zero. This results in a compressed confidence interval, which makes it less likely for a value to lie within this range.

The three replicates for fathead minnow eggs in the pump tank average 1.4% mortality with a confidence interval from 0 to 3.2% (1.4% minus and plus 1.8%). Two of the three sample averages fall within this range. For the control tank the interval is 0 to 1.4% (0.5% minus and plus 0.9%), and once again two of the three samples are within this interval. For the gravity tank the interval is 0 to 3.7% (1.8% minus and plus 1.9%), and all three samples are within this specified range.

Table 4-6. Averages of the Mean Percent Mortality Rates and 95% Confidence Intervals

Replicate	Control Tank	Gravity Drain Tank	Pumped Tank
Fathead Minnows			
1	1.7%	0.0%	53.7%
2	0.0%	0.0%	43.2%
3	0.0%	1.1%	34.8%
4	0.0%	0.0%	46.9%
5	0.0%	0.0%	45.6%
Average	0.3%	0.2%	44.8%
95% Confidence Interval	± 0.8%	± 0.5%	± 6.8%
Fathead Minnow Egg			
1	1.6%	3.7%	0.8%
2	0.0%	0.0%	3.5%
3	0.0%	1.8%	0.0%
Average	0.5%	1.8%	1.4%
95% Confidence Interval	± 0.9%	± 1.9%	± 1.8%

SECTION 5
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Appendix A:
SAMPLING AND ANALYSIS PLAN

Sampling and Analysis Plan for the Vessel General Permitting Program Pump Mortality Study

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1. INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is conducting a bench-scale study to determine fish egg and fish mortality caused by ballast water pumps on Great Lakes bulk carriers (Lakers)[1]. This study plan provides the approach that will be used to simulate ballast water discharges from Lakers, the methodology for preparing the challenge water for testing, and the procedures for collecting and analyzing living organisms for the test. The bench-scale study will be performed by EPA and EPA's technical contractor Eastern Research Group, Inc. (ERG) and ERG's subcontractor, Great Lakes Environmental Center, Inc. (GLEC). This document, in combination with the Quality Assurance Project Plan (QAPP), is intended to serve as a guide for study personnel, as well as a study review mechanism for EPA personnel.

1.1 Background

Due to a 2006 court order, EPA began permitting incidental vessel discharges from many vessels on February 6, 2009. The 2013 Vessel General Permit (VGP) (USEPA, 2013) regulates discharges incidental to the normal operation of vessels operating in a capacity as a means of transportation. The VGP includes general effluent limits applicable to all discharges; general effluent limits applicable to 27 specific discharge streams; narrative water-quality based effluent limits; inspection, monitoring, recordkeeping, and reporting requirements; and additional requirements applicable to certain vessel types.

Literature has shown that during ballast water intake, a diverse community of live organisms present in both the water column and the lake sediments is entrained into the ballast tanks (Ruiz et al, 2007). When Lakers ballast in a Great Lakes port which has been colonized by an aquatic nuisance species (ANS), and then discharge their ballast in another Great Lakes port, they have the potential to spread ANS within the Great Lakes. In Part 2.2.3.3 of the 2013 VGP, EPA included several best management practices (BMPs) for ballast water management for Lakers to reduce the likelihood of those vessels dispersing and spreading aquatic invasive species. One of those BMPs is for vessels to use their ballast pumps to empty their ballast tanks, rather than gravity draining, to produce both sheer and cavitational stresses on these organisms, theoretically resulting in higher mortality. Although pumping ballast water rather than gravity draining should result in additional organism mortality, only one study has been conducted (USCG, 2013) to support the BMP, and this study examined only larval fish and did not investigate other organisms such as zooplankton or fish eggs that can also be drawn into ballast tanks. As such, EPA is actively gathering data on the mortality caused by pumps on other types of organisms.

1.2 Objectives and General Approach

The objective of this bench-scale study is to determine if emptying ballast tanks by pumping creates greater mortality for fish eggs and fish (minnows) than emptying ballast tanks

[1] "Laker" is the common name for the large and uniquely designed and constructed dry bulk vessels (or carriers) used to transport bulk material commodities throughout the Great Lakes system. U. S. flag Lakers usually only transport goods on the four upper Great Lakes and connecting channels, as *most* are limited by their size from transiting the Welland Canal. The primary commodities transported by the Lakers include iron ore pellets, coal, grain, limestone, cement, sand, and salt.

by gravity draining. Based on the results of the bench-scale testing, additional pilot or full scale testing may be conducted under future work assignments.

The general approach of this bench-scale study will include:

- Collect fish eggs and small minnows from a laboratory-raised culture;
- Place the organisms into two process feed tanks and a control tank;
- Gravity drain one feed tank into a collection net and count the number of live and dead organisms following gravity draining;
- Pump the second feed tank into a collection net and count the number of live and dead organisms following pumping. The pumping rate will be adjusted to simulate the ballast pumping rate on a Laker;
- Determine the test handling mortality by analyzing live and dead organisms in the control tank;
- Using stastical analysis, determine the differences in mortality between the control, gravity draining and pumping for each organism type.

1.3 Bench-Scale Study Location

ERG and GLEC will conduct the bench-scale study at the Lake Superior National Estuarine Research Reserve (LSNERR) located in Superior, Wisconsin in early September, 2014. This facility was selected for the study due to its location on the shore of Lake Superior’s St. Louis River estuary (a major shipping port) and the availability of Lake Superior estuary water for maintaining the organisms before and after testing. In addition, the facility can provide laboratory and dock space needed for both the test tanks.

2. MORTALITY TESTING

This section provides the detailed procedure that will be used to conduct the bench-scale organism mortality tests at LSNERR. The bench-scale testing procedure will be divided into three phases that include: (1) constructing the test apparatus including the tanks, piping, pumps, and post treatment organism collection nets; (2) obtaining fish eggs (fathead minnow eggs) and conducting the gravity drain, pump and control testing and live/dead sample analysis; and (3) obtaining small fish (fathead minnows) and conducting the gravity drain, pump and control testing and live/dead sample analysis. Testing for each organism group (fish eggs and minnows) will be conducted separately. All tests will have multiple replicates. The following subsections describe each of these three phases in greater detail.

2.1 Bench-Scale Testing Apparatus

Figure 2-1, Figure 2-2, and Figure 2-3 are diagrams depicting the pump, gravity drain, and control system testing apparatus, respectively. Figure 2-4 is a site layout showing the arrangement of the test tanks and the sample receiving areas.

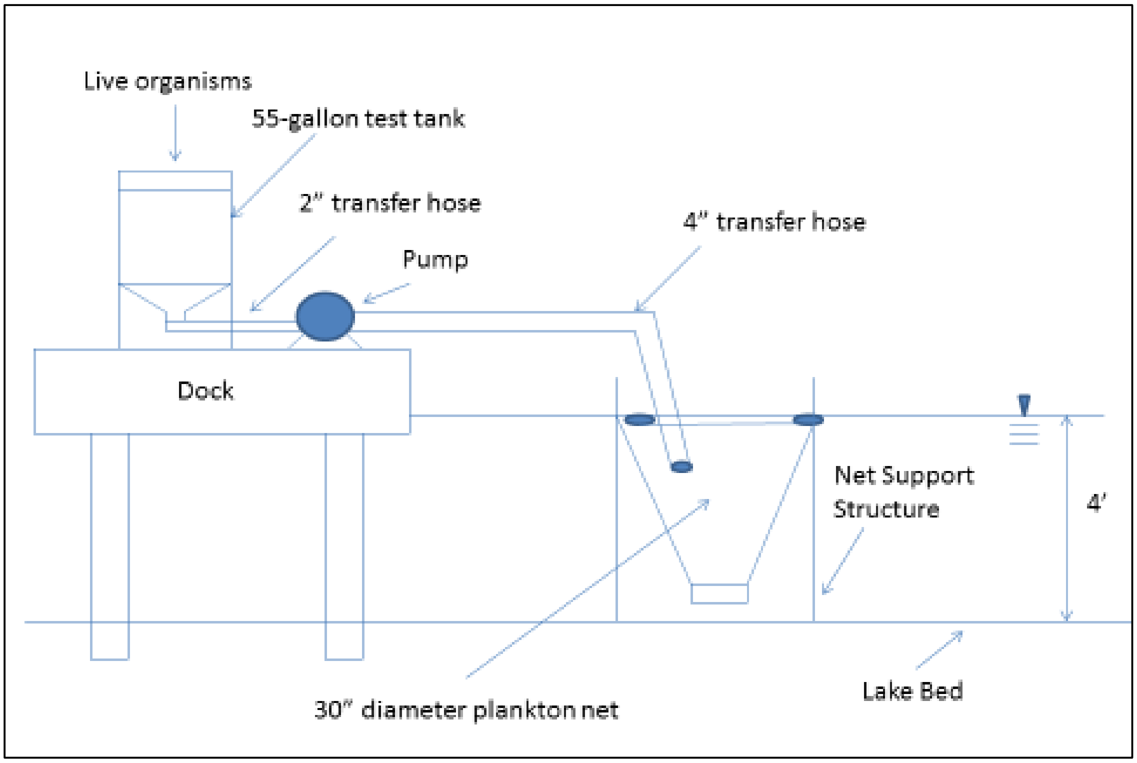

Figure 2-1. Pump System Testing Apparatus

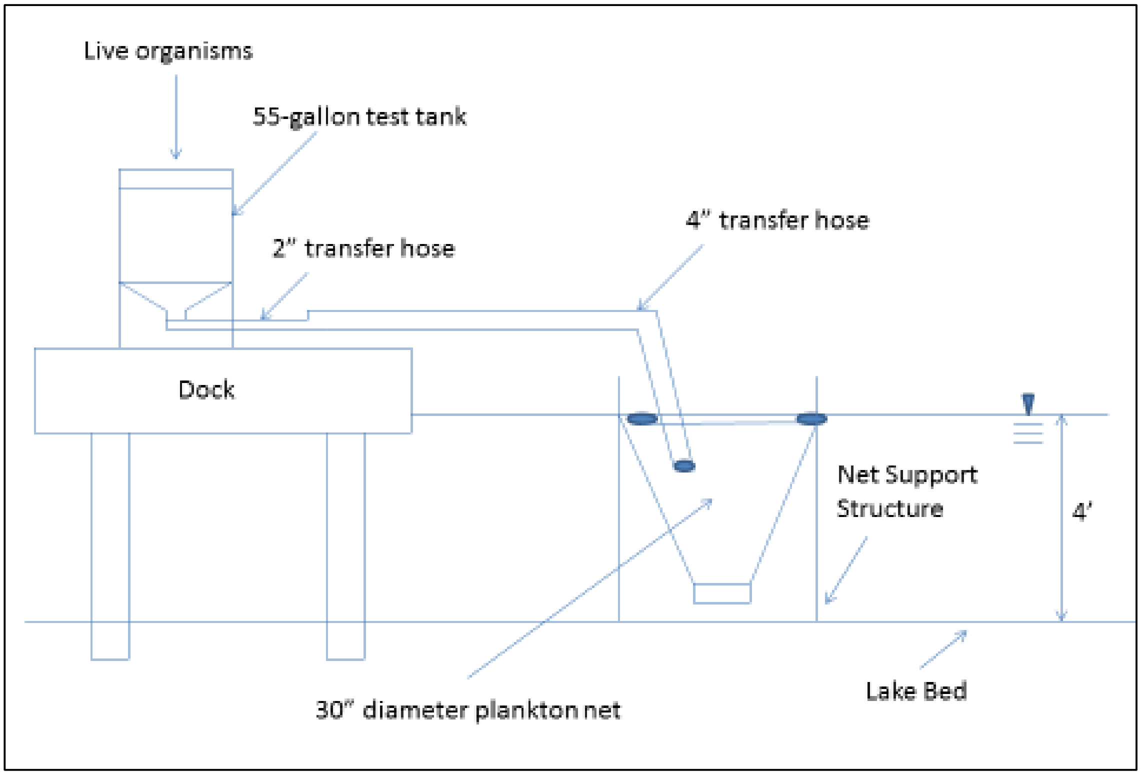

Figure 2-2. Gravity Drain System Testing Apparatus

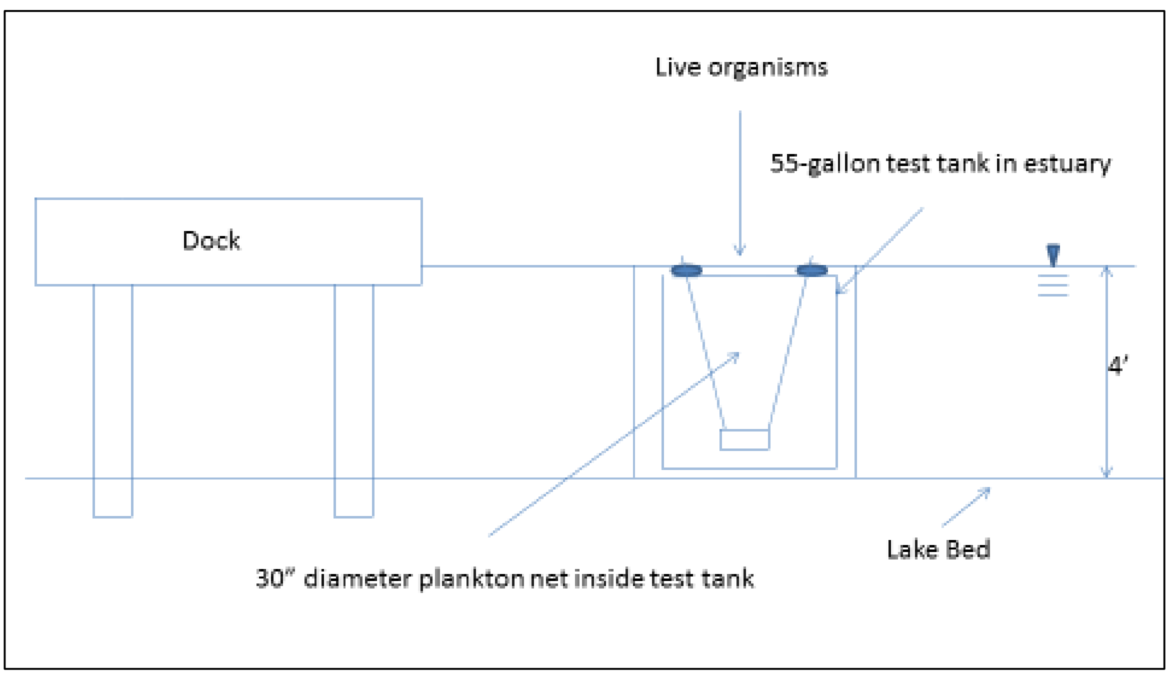

Figure 2-3. Control System Testing Apparatus

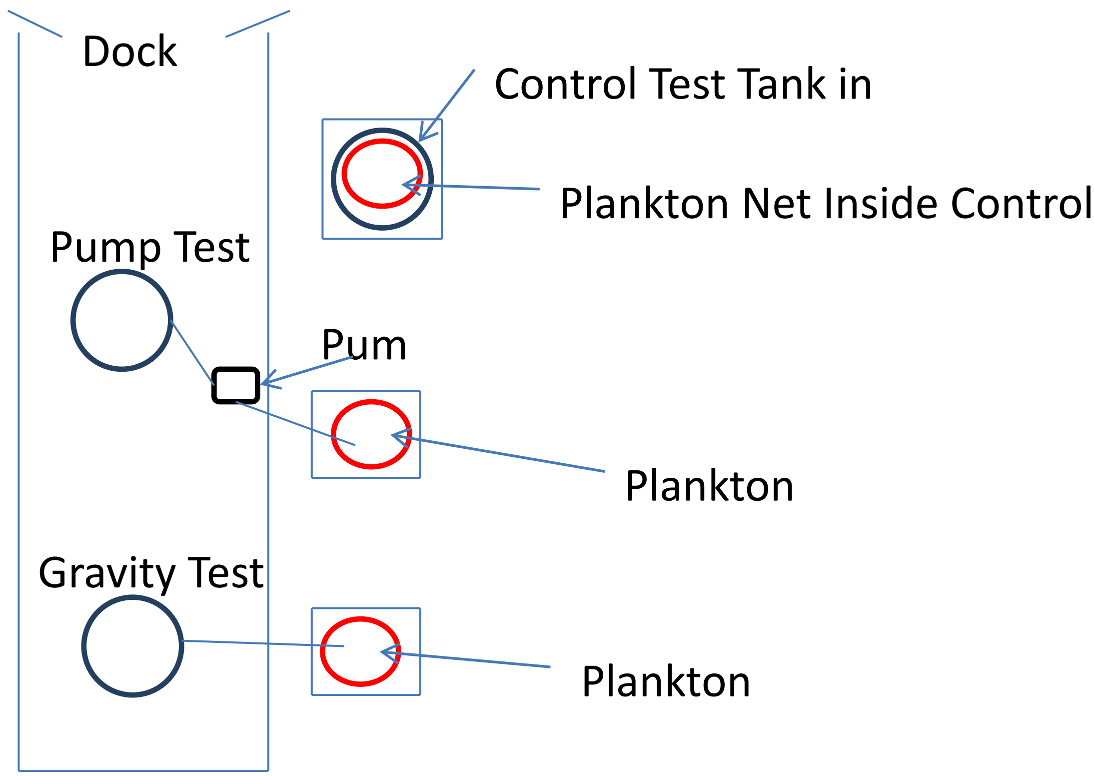

Figure 2-4. Layout of Testing System

The bench-scale testing system will consist of three 55-gallon open top plastic test tanks placed on wood stands. Each test tank has a diameter of 30 inches and a height of 35 inches. The pump and gravity drain tanks will be fitted with a 2" diameter valve beneath the tank for draining or pumping. The third tank, which will be used as a control to measure mortality caused by handling of the organisms will not be drained and therefore will not require a valve. The pump and gravity drain tanks will be filled with water directly from the St. Louis River estuary using a pump. The water will be filtered through 35 micron mesh before it enters each tank. Each organism group (fish eggs or minnows) will be placed in the water within the test tanks immediately prior to testing. The pump and gravity drain test tanks will be directed to the plankton nets placed in approximately 4' of water in the estuary. The plankton nets have a 35 micron screen size[2], are 30" diameter and will have a 1-liter cod-end cup on the bottom to capture the organisms rinsed from the nets.

The pump test tank will discharge below the water line into the plankton net to simulate a ballast discharge through the sea chest. The gravity drain test tank will also discharge into the plankton net below the water line to simulate gravity draining by Lakers.[3] ERG decided to place the plankton nets in the estuary rather than another receiving tank to buffer the force of the water and minimize the potential mortality that could be caused by the organisms contacting plankton

[2] U.S. Environmental Protection Agency Environmental Technology Verification (ETV) Program. Generic Protocol for the Verification of Ballast Water Treatment Technology. Section 5.4.6.4 requires the use of a 35 micron screen size for capture of zooplankton.

[3] According to Mr. Mr. Jason Toast with The Interlake Steamship Company and Mr. Jim Weakly at the Lake Carriers Association, American Lakers discharge ballast water through either their low or high sea chests below the water line.

nets during discharge. In addition, to further reduce mortality caused by organisms being forced into the plankton nets by water pressure, the pipe diameter on the pump discharge will be increased from 2" to 4". This will reduce the pressure and force with which test water will be discharged into the plankton net to help counteract the force imposed by the pump. The gravity drain tank will also include a pipe size increase from 2" to 4" near the middle of the pipe run in to ensure consistency between the pump and gravity test systems. To ensure against "contamination" of the test net with organisms in the water (and escape of live organisms inside the test nets), net supports will be constructed to hold nets 12 inches above the water level. Once the pump and gravity drain test tanks are empty, a 1-hour recovery period will occur[4], after which the plankton nets will be gently lifted out of the receiving areas and the organisms will be collected for analysis by GLEC.

To evaluate organism mortality caused by handling, the control tank (3rd tank) will be submerged in the estuary adjacent to the pump and gravity drain collection nets. The control tank will be lined with a 35 micron plankton net. The control tank will be submerged rather than drained to reduce possible organism mortality that could be caused by the change in elevation from the dock to the sample receiving area in the estuary. Water in the control tank will also be filtered through 35 micron mesh, the same as water in the gravity and pump test tanks. Test organisms will be introduced into the control tank and its plankton net at the same time that test organisms are introduced into the test tanks on the dock. This net will also be supported 12 inches above the water level to guard against organism escape or entrainment of additional organisms from estuary water. The control tank plankton net will be raised at the same time as the nets from the gravity drain and pump test nets and the mortality of the organisms collected in the control tank net will be evaluated.

To simulate ballast pump conditions from a Laker, ERG will use a Honda trash pump with 2" diameter hose connected to the valve on the bottom of the pump test tank. Based on data provided in the literature, ballast flow through the piping of a Laker is 10 ft/second; therefore, flow from the pump should range between 1,380 and 2,450 gal/hour with an average of 1,920 gal/hr (32 gal/min).[5] Because the power of the water pressure coming out of a 2" diameter hose from the pump would be high enough to cause mortality in test organisms by forcing them at high velocity into the plankton net, the diameter of the discharge hose will be increased to 4", thus reducing the pressure by a factor of 2 and reducing undesirable mortality caused by the sampling procedure rather than the pump.

2.2 Fish Egg Mortality Testing Procedure

If live fathead minnow eggs are available from EPA's Mid-Continent Ecological Division (MED) laboratory in Duluth for testing in early September, mortality testing with this organism will be conducted. Fathead minnows were selected for testing because they are either native to Lake Superior and the St. Louis River. Eggs will be acclimated to estuary water by bringing them to test water temperature at a rate of no more than 5^{o}C per hour. Hatchery water

[4] Great Ships Initiative Standard Operating Procedure GSI/SOP/LB/RA/SA/2 Procedure for Zooplankton Sample Analysis, July 2009.

[5] Cangelosi, A., Schwerdt, T., Mangan, T., Mays, N., and Prihoda, K., A Ballast Discharge Monitoring System for Great Lakes Relevant Ships: A Guidebook for Researchers, Ship Owners, and Agency Officials, Table 1, November 2011.

hardness will also be obtained and compared to estuary water to ascertain whether test water will need to have its hardness increased with well water or mineral water to ensure the osmotic integrity of the eggs.

Each of the three test tanks will be filled with filtered (35 micron) St. Louis River estuary water and dosed with enough eggs to provide a concentration of approximately three eggs per gallon. For three 55-gallon test tanks and four replicates, approximately 2,000 fathead minnow eggs will be needed to complete the study. Four replicates rather than 5 will be conducted for fish eggs due to their limited availability from MED. For each test, the eggs will be introduced into the three tanks (gravity drain, pump and control test tanks) at the same time. Because eggs will already be at test-water temperature, no acclimation period is necessary or desirable because the test tanks on the dock will heat up in the September sun, increasing stress on the eggs. Once the eggs pass from the gravity drain and pump test tanks into the plankton nets suspended in the sample receiving areas established in the estuary, the one hour recovery period will being. Following this, the plankton nets will be raised to capture the eggs in the cod-end collection container for analysis of mortality. Mortality assessment will be conducted under dissecting microscopes and will be determined by the presence of a heartbeat or larval movement in each egg. Egg condition will be noted as appropriate.

2.3 Fish Mortality Testing Procedure

Small fathead minnows or other similar bait fish will be obtained from a local bait supplier in Wisconsin and used to test mortality of small fish passing through a pump. Fathead minnows or other bait fish were selected for testing because they are either native to Lake Superior and the St. Louis River, or their populations have already been established. These minnows also tend to be relatively hardy, helping to ensure that there will be little handling and control mortality. Minnows will be acclimated to estuary water by bringing them to test water temperature at a rate of no more than 5°C per hour. Culture water hardness will also be obtained and compared to estuary water to ascertain whether test water will need to have its hardness increased with well water or mineral water to ensure no undue osmotic stress on the minnows.

Each 55-gallon test tank will be filled with St. Louis River estuary water and dosed with approximately 100 minnows. For three test tanks and five replicate tests, approximately 1,500 minnows will be required for the study. Minnows will be introduced into the three test tanks at the same time and allowed five minutes to acclimate (a long acclimatization period is not desirable due to the difficulty of keeping test tank water from heating up; minnows will already acclimated to test water temperature by the time the test starts). Test tanks will then be pumped or drained into the plankton nets established in the sample receiving areas in the estuary. Once minnows are pumped or gravity drained from the test tanks into the sample receiving area plankton nets, the one hour recovery period will begin. Following this, field personnel will raise the nets and collect the organisms to evaluate mortality. Mortality assessment will be conducted by GLEC personnel either under the microscope or with the naked eye by assessing body or gill movement depending on fish size. Notes will be made on fish body condition as appropriate.

3. SAMPLE COLLECTION AND ANALYSIS METHODS

This section describes the sampling procedures and analytical methods that will be used by the study team to determine organism mortality in the control, gravity drain, and pump test tanks.

3.1 Organism Sampling Procedures

The following sampling procedures will be used to raise the plankton nets from the sample receiving areas in the estuary and collect the organisms for mortality analysis.

3.1.1 Fish Egg Sampling Procedure

Plankton nets containing fish eggs will be individually raised using their tow harnesses by personnel standing on the dock. Because all water from the tests will be released into or contained within the nets, all eggs used in each test will be contained within the net for each test (control, pump, or gravity drain). As each net is raised, personnel in the water will gently wash down the sides of the net to wash eggs down into the collection container. Once all eggs have been washed into the collection container of each net, the containers will be removed and carried to the lab area for microscopic analysis. In the lab area, the containers will be placed into a cooler with water, which will be maintained at estuary water temperature using ice.

3.1.2 Fish Sampling Procedure

Plankton nets containing minnows will be individually raised using their tow harnesses by personnel standing on the dock. Because all water from the tests and control will be released into or contained within the nets, all fish used in each test will be contained with the net for each test (control, pump, or gravity drain). As each net is raised, personnel in the water will wash down the sides of the net as needed to wash minnows down into the collection container (depending on fish size, several containers may be used to avoid overcrowding the fish). Once all fish have been washed into collection containers from each net, the containers will be carried to the lab area for microscopic analysis. In the lab area, containers from each test will be decanted into 10-gallon aquaria (one aquarium for each test: control, pump, and gravity drain). Aquaria will be maintained at estuary water temperature using ice; dissolved oxygen levels will be maintained using aerators.

3.2 Organism Analysis Procedures

Samples collected in the 1-liter containers from the cod end of the plankton nets will be immediately analyzed in the LSNERR on-site laboratory by GLEC laboratory staff. Table 3-1 shows the total number of samples that will be collected and analyzed for each organism type. Standard operating procedures (SOPs) that will be used by field laboratory personnel for organism mortality assessments are provided in Appendix A. Samples will be hand-carried from the sample receiving areas directly to the laboratory. Containers will be capped with plankton netting and placed in coolers containing estuary water that is aerated with bubblers and whose temperature is regulated using ice to maintain estuary water temperatures. This will minimize mortality due to crowded holding conditions before mortality assessment can be completed.

Table 3-1. Summary Number of Samples, Sample Bottles, Preservation, and Holding Time Requirements

Parameter	Estimated Number of Samples	Sample Bottle and Volume	Preservation	Holding Time
Fish Eggs	12	1-liter container from cod end of plankton net.	Maintain temperature and DO levels.	Immediate
Fish (minnows)	15	1-liter container from cod end of plankton net.	Maintain temperature and DO levels.	Immediate

The following subsections provide additional details on how samples will be analyzed in the on-site laboratory for live/dead fish eggs and minnows.

3.2.1 Fish Egg Mortality Assessment

Eggs will be removed from each container for microscopic examination by gently swirling each container to re-suspend any eggs that have settled to the bottom and then using a large pipette to place an approximately 20 milliliter subsamples into a counting chamber. This process will be repeated 30 - 50 times (600 - 1000 mL from a 1000 mL container) until 60 minutes have elapsed while the nets and tanks are cleaned and re-set for the next replicate test run. With four personnel concurrently examining eggs under microscopes, about eighty percent of the eggs from each net (130 eggs) should be able to be assessed for mortality between test replicates.

Egg mortality will be assessed under dissecting microscopes by either viewing a heartbeat or by seeing larval movement inside each egg, depending on the age of the egg. If the egg is so undeveloped that it does not have a visible heart, it will be recorded as “indeterminate”. If the egg contains a visible larva that is not moving, the egg will be gently prodded with very fine forceps in an attempt to elicit a response. Egg larvae that do not respond to prodding will be recorded as dead. Thus, data collection will consists of counts of live, dead, and “indeterminate” eggs. Percent mortality calculations will use only live and dead counts. All eggs will be preserved separately from each net so that a total count of eggs can be generated for each replicate.

3.2.2 Fish Mortality Assessment

Minnows will be held in 10-gallon, temperature-monitored, aerated aquaria (one for each test type: pump, gravity drain, control) to insure against post-test mortality. Minnows will be gently removed from each aquarium using a fine-mesh aquarium net and placed in an appropriately-sized dish for examination (whether examination is by the naked eye, a magnifying glass, or a microscope will depend on minnow size). With three personnel examining minnows, GLEC personnel should be able to examine nearly all the minnows used in each test.

Mortality assessment will be based on movement or, if none, gill movement. Intact but non-moving minnows will be gently prodded. Notes will be made on body condition of the minnows as appropriate (e.g., if individuals are noticeably abraded or only pieces of minnows are found). If minnows have been chopped apart by the pump, only heads will be counted for the mortality count. Data collection will consist of counts of live and dead minnows. A separate category of “injured” minnows may also be counted, but this will be a completely separate count, so that injured/damaged minnows are also counted as either alive or dead. Percent mortality calculations will use only live and dead counts; counts of injured/damaged minnows will be reported separately (but not used in the percent mortality calculations) to help assess cause of death by pump propeller. Percent mortality will be calculated as a percentage of dead individuals out of the total count in each test replicate.

3.3 Sample Labeling

Each sample container will be coded with a unique sample number and labeled at the time of collection. Samples will be labeled with the replicate number (1, 2, or 3) and if the sample is from the pump, gravity drain or control test tank discharge. For example, a sample for fish eggs collected from the pump discharge during the second replicate test would be labeled “Egg Pump Rep 2, sample 1 of 1” (if only one container per replicate).

3.4 Chain of Custody

Due to the extremely short holding times for the organism samples, individual Chain of Custody reports (CCRs) will not be prepared prior to each sample being delivered to the laboratory. Instead, the person delivering the sample to the laboratory will log the sample information on a CCRs being maintained in the laboratory. A CCR will be developed for each replicate test for each organism group. The CCR will remain in the laboratory and the individual delivering the sample will be responsible for logging the sample information including the sample number, analysis to be performed (e.g., live/dead fish eggs), time and date of sample collection, and the initials of the person delivering the sample to the laboratory.

4. QUALITY ASSURANCE FOR FIELD SAMPLE ANALYSIS

Quality assurance/quality control (QA/QC) procedures applicable to this study are outlined in the QAPP (ERG, 2014). The QA/QC program includes the components discussed in the following subsections.

4.1 Documentation of Sample Custody

All samples will be delivered to the on-site laboratory by a member of the study team. While samples are being collected, samples and sampling equipment will be maintained in the physical possession or view of at least one member of the sampling crew. To maintain a record of sample custody, the sampling crew will complete a CCR form for each study replicate. These CCR forms will be used to document sample custody transfer from the field to the on-site laboratory.

4.2 Field Replicates

A total of 5 mortality tests (pump, gravity drain, and control) for fish eggs and fish (fathead minnows) will be conducted. The average percent mortality in the control for each replicate will be compared to the average percent mortality of the pumped and gravity drain tests to determine if the gravity and pumped tests have greater mortality than the control. Variability between the control replicates for each organism will be measured as a standard deviation that will be used to determine an upper bound control mortality. Average mortality in the pumped and gravity tests, along with variability measured as a standard deviation between replicates, will be used to determine a lower bound control mortality. The average and upper bound control mortality will be compared to the average and lower bound control mortality of the pump and gravity tests to determine if the pump and gravity tests caused more mortality than the control.

4.3 Laboratory Duplicates

GLEC anticipates analyzing half or more of all organisms in each net for each test replicate. For eggs, this will entail counting 30 - 50 twenty milliliter subsamples in the counting chamber for analysis of live/dead organisms. Live/dead analysis will be made by counting organisms under the dissecting microscope (or magnifying glass or naked eye for fathead minnows). During analysis, ten percent (3 to 5) of the subsamples will be re-counted by a second observer. The second live/dead count designated for duplicate analysis will be conducted immediately following the initial count. Results from both counts will be recorded and the values used to calculate a relative percent difference.

5. TESTING ACTIVITIES

This section of the plan summarizes the ERG and GLEC sampling team organization, pre-visit preparation, field sampling activities, and logistics including site contacts and site location.

5.1 Study Team Organization

Mr. Mark Briggs will serve as ERG's on-site project manager. He will be assisted by Ms. Kathleen Wu, also with ERG. ERG will be responsible for procuring test tanks and plankton nets, renting the pumps and associated transfer hoses, and procuring the supplies (extra cod-end containers for the plankton nets, lumber to build support structures, fish tanks with aerators, etc.) to establish the collection areas in the St. Louis River Estuary. ERG is also responsible for procuring fathead minnow eggs and small minnows for testing, and for procuring ice to maintain test tank temperatures. During the field study, ERG will be responsible for calibrating the pump speed and/or flow rate, filling the test tanks with estuary water prior testing, adding organisms to the test tanks, and pumping or gravity draining the test tanks to the plankton nets.

Mr. Chris Turner will serve as GLEC's Principal Investigator (PI). He will be assisted by two additional GLEC field and microscopy lab technicians who will work primarily on mortality assessment. Mr. Turner will oversee and assist several aspects of the mortality assessment and mortality detection limit assessments. GLEC will provide microscopes, forceps, sub-sampling gear, and counters.

5.2 Pre-Visit Preparation

Prior to conducting the field study, the ERG crew chief and the GLEC PI will distribute this Sampling and Analysis Plan (SAP), the QAPP, and the Health and Safety Plan (HASP) to each team member and ensure they are completely familiar with the sampling, quality, and health and safety requirements. The ERG crew chief will also provide LSNERR site personnel copies of the SAP and any site-specific supplemental information prior to the start of sampling.

The crew chief will also coordinate the procurement and shipment of all necessary sampling and health and safety equipment.

5.3 Field Testing Schedule

The field study is tentatively scheduled for early September 2014. Prior to equipment setup and testing, the crew chief will notify ERG's Health and Safety Coordinator of any revised activities along with recommended revisions to the proposed health and safety procedures. Together, they will review the proposed health and safety procedures, incorporate any site-specific changes indicated by the Health and Safety Coordinator, and obtain approval for sampling from the Health and Safety Coordinator before proceeding with testing activities.

Table 5-1 and Table 5-2 show potential timelines for the fish (minnows) and fish eggs test days. Equipment will be setup and tested the day prior to beginning the study (Monday). Setup will include establishing the laboratory area where mortality observations will be made, placing the test tanks and pump on the dock, constructing the plankton net support structures, plumbing the system, and testing the system using ambient water. At the end of the setup day, equipment will be cleaned and readied for minnow testing which will begin the next day.

Fish (minnow) testing is expected to be conducted the first day of the study (Tuesday), followed by fish egg testing on the second day (Wednesday). At the end of testing, the tanks, pumps, piping, plankton nets, and laboratory equipment will be removed from LSNERR and returned.

Table 5-1. Fish (Minnows) Daily Testing Schedule

Time	Fish Testing (Day 1)
0730	ERG arrives at LSNERR and readies tanks and establishes receiving nets in the water.
0800	ERG arrives at LSNERR with fathead minnows from the Wisconsin bait supplier and places minnows in aerated, temperature-monitored storage tank. Minnows are acclimated to test temperature water at a rate of no more than 5^{o}C per hour. GLEC arrives and collects a water sample to verify water hardness, conductivity, temperature and dissolved oxygen levels are appropriate for the minnows.
0830	ERG fills tanks with filtered estuary water, adds approximately 100 fathead minnows to each test tank, and allows to acclimate for 5 minutes.
0835	Replicate 1 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
0935	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 1 sample containers delivered to on-site laboratory for mortality assessment.
1030	Plankton nets cleaned and reestablished for Replicate 2. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water and 100 fathead minnows added to each test tank and allowed to acclimate for 5 minutes.
1035	Replicate 2 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1135	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 2 sample containers delivered to on-site laboratory for mortality assessment.
1300	Plankton nets cleaned and reestablished for Replicate 3. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water and 100 fathead minnows added to each test tank and allowed to acclimate for 5 minutes.
1305	Replicate 3 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1405	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 3 sample containers delivered to on-site laboratory for mortality assessment.
1500	Plankton nets cleaned and reestablished for Replicate 4. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water and 100 fathead minnows added to each test tank and allowed to acclimate for 5 minutes.
1505	Replicate 4 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1605	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 4 sample containers delivered to on-site laboratory for mortality assessment.
1700	Plankton nets cleaned and reestablished for Replicate 5. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water and 100 fathead minnows added to each test tank and allowed to acclimate for 5 minutes,
1705	Replicate 5 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1805	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 5 sample containers delivered to on-site laboratory for mortality assessment.

Table 5-1. Fish (Minnows) Daily Testing Schedule

Time	Fish Testing (Day 1)
1930	Fish testing complete and plankton nets cleaned and reestablished for Day 2 testing with fish eggs.

Table 5-2. Fish Egg Daily Testing Schedule

Time	Fish Egg Testing (Day 2)
0800	ERG arrives at LSNERR and readies tanks and establishes receiving nets in the water.
0830	GLEC crew arrives at LSNERR. ERG arrives at LSNERR with fathead minnow eggs from EPA-MED and places eggs in aerated, temperature-monitored storage tank. Eggs are acclimated to test temperature water at a rate of no more than 5^{o}C per hour. GLEC collects a water sample for analysis of ambient water parameters (hardness, conductivity, temperature and DO).
0930	ERG fills tanks with estuary water, adds approximately 165 fathead minnow eggs to each test tank, and allows to acclimate for 5 minutes.
0935	Replicate 1 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1035	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 1 sample containers delivered to on-site laboratory for mortality assessment.
1130	Plankton nets cleaned and reestablished for Replicate 2. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water. ERG places approximately 165 fathead minnow eggs in test tanks for acclimation for Replicate 2.
1135	Replicate 2 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1235	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 2 sample containers delivered to on-site laboratory for fish egg mortality assessment.
1330	Plankton nets cleaned and reestablished for Replicate 3. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water. ERG places approximately 165 fathead minnow eggs in test tanks for acclimation for Replicate 3
1335	Replicate 3 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1435	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 3 sample containers delivered to on-site laboratory for mortality assessment.
1530	Plankton nets cleaned and reestablished for Replicate 4. Pump, gravity drain, and control test tanks cleaned and refilled with filtered estuary water. ERG places approximately 165 fathead minnow eggs in test tanks for acclimation for Replicate 3.
1535	Replicate 4 - pump, gravity drain and control test tanks discharged into plankton nets and 1 hr recovery period begins.
1635	Plankton nets lifted for the pump, gravity drain and control test tank discharges and Replicate 4 sample containers delivered to on-site laboratory for fish egg mortality assessment.
1800	Plankton nets cleaned and stowed. Fish egg testing complete.

5.4 Logistics

This subsection summarizes the field study team personnel, site contacts, EPA contacts and address, and ERG project management contact and address.

5.4.1 Field Study Team Contacts

Mark Briggs (Crew Chief)
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6. DATA ANALYSIS

Mortality analysis data will be recorded on the datasheets for each of the organism types. Data sheets for fish eggs and minnows are provided in Figures 6-1 and 6-2. Following completion of testing and data quality analysis, the qualified data will be transferred from the datasheets to Excel spreadsheets. A separate Excel workbook containing mortality data will be established for fish eggs and minnows. Within each workbook, tabs will be created for the control, gravity drain, and pump mortality data. Data will include the number of live and dead organisms counted in each plankton net container collected from the individual plankton nets retrieved. The spreadsheet will calculate an average count of live and dead organisms from each plankton net retrieved for each test replicate. Duplicate QC count results and relative percent differences will also be calculated and reported. ERG and GLEC will analyze the data by comparing organism mortality in the control to organism mortality caused by gravity draining and pumping for each organism type. Ultimately, percent mortality will be statistically tested for significant differences among pump, gravity drain, and control data for each organism type.

Counter ______________________________ Date __________________________

Test Type: Control Pump Gravity

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Figure 6-1. GLEC Field Laboratory Mortality Data Sheet – Fish Eggs

Counter ______________________________ Date _________________________

Replicate _______ **Test Type:** **Control Pump Gravity**

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Replicate ___

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	QA Check	Total
Dead												
Alive												

Damage Notes:

Figure 6-2. GLEC Field Laboratory Mortality Data Sheet - Minnows

7. RELATED BIBLIOGRAPHY

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Appendix A:

STANDARD OPERATING PROCEDURES FOR LABORATORY-BASED VIABILITY ANALAYSIS OF FISH EMBRYOS AND LARVAE FOR TOXICITY TESTING AND OTHER SPECIAL ENVIRONMENTAL STUDIES AND PURPOSES

PROCEDURE FOR CONDUCTING LABORATORY-BASED ANALYSIS OF FISH EMBRYOS AND LARVAE VIABILITY FOR TOXICITY TESTING AND OTHER SPECIAL ENVIRONMENTAL STUDIES

Method Reference: ASTM E 1241-98. 1999. Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. ASTM, Volume 11.05.

September 8, 2014

Great Lakes Environmental Center, Inc.
(GLEC)

Tyler K. Linton — 9/8/14
Tyler Linton — Date
Technical Author

9/8/14
Chris Turner — Date
User Reviewer

M. M. DeGraeve — 9-8-14
Mick DeGraeve — Date
GLEC Management

J. A. Hansen — 9/8/14
Jennifer Hansen — Date
GLEC Quality Assurance Officer

Training Statement:
I have read, understand, and agree to follow this SOP.

Signature ______________________ Date ____________

Printed Name ______________________

TABLE OF CONTENTS

I. SCOPE/PURPOSE

1.1 This procedure describes the methodology for conducting viability (live/dead) analysis of fish embryos and larvae used for aquatic toxicity testing and other environmental studies, such as special fish embryo-larval studies or entrainment studies. The emphasis is on fathead minnows (*Pimephales promelas*), but the method is adaptable to other related minnow or small fish species.

1.1.1 During early-life stage chronic toxicity testing and certain other tests and studies fish embryos are continuously exposed for days or weeks to selected concentrations of the test material, with observations of embryonic development, hatching, survival and growth being recorded.

1.1.2 Tests are typically initiated with embryos between 2 and 24-hrs old, and generally must be less than a defined age, e.g., 48-hrs old for early-life stage toxicity testing.

1.1.3 Minnow species such as *P. promelas* are available from commercial sources or in-house laboratory cultures.

1.1.4 The development, hatching, growth and survival of fertilized eggs and larvae is used to determine chronic toxic effect concentrations and other unacceptable or adverse impact levels reflective of chemical effects or other types of specific biological and physical effects such as tissue contaminant loading an entrainment.

1.2 Experience with fish embryos and larvae or training with someone with such experience is required before using this SOP with analytical samples.

II. SUMMARY OF METHOD

2.1 The procedure to observe the embryos for viability consists of dividing them in sub-samples, taking each of them out of the exposure or other temporary container, and examining them under a microscope for viability status and/or other irregularities.

2.2 The procedure to observe larvae or small fish for viability consists of visually verifying that each individual in the test, whether in an exposure treatment replicate of an aquatic toxicity test or in a different container representing a unique treatment/test group from a special study, is viable and/or otherwise free of physical abnormalities or irregularities in appearance.

III. DEFINITIONS

3.1 Embryo – a fertilized egg.

3.2 Entrainment - to pull or draw in and transport.

3.3 Fungused – organisms (usually embryos) infected with waterborne fungus.

3.4 Operculum - the hard bony flap covering and protecting the gills of a fish.

3.5 Viability – alive based the observance of heartbeat, opercular movement, and the ability to respond to stimulus (a gentle touch with a laboratory pipette tip).

IV. INTERFERENCES AND CAUTIONS

4.1 Removal of fungused embryos should be done quickly, and the remaining viable embryos returned to the incubation tanks as quickly as possible so that they are not damaged by desiccation.

4.2 It is recommended to maintain organisms in the incubation/holding tanks at the same environmental conditions at which embryos/fish have been exposed during spawning, or while being reared.

4.3 The water temperature in the rearing tanks is allowed to follow ambient laboratory temperatures of 20-25ºC, but sudden, extreme, variations in temperature must be avoided.

4.4 To prevent unnecessary osmotic stress it is recommended that while larval and other small-sized fish should not be subjected to more than a 50 mg/L (as $CaCO_3$) change in water hardness in any one 24-h period; acclimation to more than a 100 mg/L in total is acceptable.

4.5 Equipment used to handle embryos must be sterilized (soap and hot water and well rinsed at a minimum), and hands should be washed before and after handling.

V. HEALTH AND SAFETY

5.1 Regard each chemical/reagent as a potential health hazard, and read the MSDS for each before starting work.

VI. EQUIPMENT AND SUPPLIES

6.1 Reflected-light (dissecting) stereo microscope;

6.2 Illuminator box(es);

6.3 Watch glass(es)**;**

6.4 Petri dish(es);

6.5 Magnifying glass(es);

6.6 Forceps;

6.7 Blunt probe;

6.8 Pipette(s) with bulb(s);

6.9 Plastic squirt bottle(s);

6.10 Plastic sample and other containers (as required);

6.11 Meter to check hardness; and

6.12 Thermocouple thermometer to monitor temperature during incubation.

VII. REAGENTS AND STANDARDS

7.1 Gases – Not Applicable. No gases are used in this procedure.

7.2 Reagent Water – Not Applicable. No reagent water is used in this procedure.

7.2.1 Deionized (DI) or Dechlorinated water for rinsing specimens

7.3 Reagents – Not Applicable. No reagents are used in this procedure.

7.3.1 70% Ethanol (when preserving specimens is required).

7.4 Standard Solutions – Not Applicable. No standard solutions are used in this procedure.

7.5 Biological Specimens – The only biological specimens are those used in this procedure to determine effects (i.e., the analytical samples).

VIII. SAMPLE COLLECTION PRESERVATION AND STORAGE

8.1 Sources

8.1.1 Commercial Sources. Small fish (such as the fathead minnow), juveniles and adults are available from commercial biological supply houses or laboratory culture facilities. Fish obtained from outside sources for use in toxicity or other tests may not always be of suitable age and quality. Fish provided by supply houses should be guaranteed to be: (1) the correct species, (2) disease free and in good condition, and (3) in the requested age range. The latter can be ascertained by obtaining a record of the date on which the eggs were deposited.

8.1.2 In-house Culture. Suitability of fish for use in toxicity testing and for other scientific testing purposes can be assured by developing an in-house culture. Fathead minnows are particularly suited to in-house culture because they are: 1) common and widely distributed, and therefore adults for brood stock are easy to obtain; 2) their life history is well known, and they are relatively hardy and easy to reproduce and maintain in good condition in the laboratory or culture facility; and 3) embryos can be available throughout the year and are less likely to be diseased.

Note: Because the quality of embryos and/or fish represents a crucial factor for hatching and study success and for production of test organisms of good quality, any stress to embryos and fish should be avoided, such as: physical shock during transport, acclimation, and/or incubation; thermal or osmotic shock. It is recommended that organisms are maintained in the incubation/holding tanks at the same environmental conditions at which embryos/fish have been exposed during spawning or while being reared.

8.2 Embryo Incubation, Acclimation and Handling

8.2.1 Incubation. There are three primary methods for incubating fathead minnow (and other minnow) embryos obtained from outside or internal sources: on substrates, in a separatory funnel, or in embryo incubation cups (see USEPA, 2002). After fertilization, fathead minnow embryos are approximately 1.2 mm to 1.6 mm in diameter. The incubation time depends on temperature, and is 4.5 to 6 days at 25ºC.

8.2.2 Incubation on substrates. Several (2-4) tile substrates are placed on end in a circular pattern (with the embryos on the inner side) in 10 cm of water in a tray. The tray is then placed in a constant temperature water bath, and the embryos are aerated with a 2.5 cm air stone placed in the center of the circle. The embryos are examined daily, and the dead and fungused embryos are counted, recorded, and removed with forceps. At an incubation temperature of 25ºC, 75-100% the embryos hatch in five days. At 22ºC, embryos incubated on aerated tiles require seven days for 50% hatch.

8.2.3 For additional details regarding spawning, fertilization and embryo incubation on substrates, see GLEC SOP TOX 0006 *Culturing Pimephales promelas*.

8.2.4 Incubation a in a separatory funnel. The embryos are removed from the substrates with a gentle circular rolling action of the index finger ("rolled off") (Gast and Brungs, 1973), their total volume is measured, and the number of embryos is calculated using a conversion factor (for fathead minnows) of approximately 430 embryos/mL. The embryos (approximately 1500 to 2000) are incubated in about l.5 L of water in a 2 L separatory funnel maintained in a water bath. The embryos are stirred in the separatory funnel by bubbling air from the tip of a plastic micro-pipette placed at the bottom, inside the separatory funnel. During the first two days, the embryos are taken from the funnel daily, those that are dead and fungused are removed, and those that are alive are returned to the separatory funnel in clean water. The embryos hatch in four days at a temperature of 25ºC. However, usually on day three the eyed embryos are removed from the separatory funnel and placed in water in a plastic tray and gently aerated with an air stone. Using this method, the embryos hatch in five days.

Note: With this incubation method, hatching time is greatly influenced by the amount of agitation of the embryos and the incubation temperature. If on day three the embryos are transferred from the separatory funnel to a static, un-aerated container, 50% of the embryos will hatch in six days (instead of five), and a l00% will hatch in 7 days.

8.2.5 Incubation in incubation cups. The embryos are "rolled off" the substrates, and the total number is estimated by determining the volume. The embryos are then placed in incubation cups attached to a rocker arm assembly (Mount, 1968). Both flow-through and static renewal incubation can be used. On day one, the embryos are removed from the cups and those that are dead and fungused are removed. After day one, only dead embryos are removed from the cups. Most of the embryos will hatch in five days if incubated at 25ºC.

8.2.6 Acclimation. In general, embryos should be standing in dilution water within 3ºC of the desired test temperature. To acclimate, embryos should not be subjected to more than a 3ºC change in water temperature in any one 12-h period, and preferably not more than 3ºC in 72 hr (ASTM, 2013). The concentration of dissolved oxygen should be maintained between 60 and 100% saturation. To prevent unnecessary osmotic stress, embryos should not be subjected to more than a 50 mg/L (as $CaCO_3$) change in water hardness in any one 24-h period, and preferably not more than a 100 mg/L hardness change in total.

Note: during the incubation and acclimation period, the embryos are examined daily for viability and fungal growth, until they hatch. Unfertilized eggs and embryos that have become infected by fungus should be removed with forceps using a table top magnifier-illuminator (see *Microscopic Viability Observations and Viability Analysis of Embryos*, below). Non-viable eggs become milky and opaque, and are easily recognized. The non-viable eggs are very susceptible to fungal infection, which may then spread throughout the egg mass. Removal of fungused embryos should be done quickly, and the remaining good embryos returned to the incubation tanks as quickly as possible so that they are not damaged by desiccation.

8.2.7 Handling. Embryos should be handled as little as possible. When handling is necessary, it must be done gently, carefully and quickly so that the organisms are not unnecessarily stressed. Smooth bore glass tubes are best for handling and transporting embryos.

8.2.8 Equipment used to handle embryos must be sterilized (soap and hot water and well rinsed at a minimum), and hands should be washed before and after handling.

8.3 Larval and Small Fish Rearing/Holding and Acclimation and Handling

8.3.1 Rearing/Holding. Newly-hatched larvae are transferred daily from the egg incubation apparatus to small rearing tanks, using a large bore pipette, until the hatch is complete. New rearing tanks are set up on a daily basis to separate fish by age group. Up to 1500 newly hatched larvae can be placed in a 60 L (15 gal) or 76 L (20 gal) all-glass aquarium for 30 days. A density of 150 fry per liter is suitable for the first four weeks. The water temperature in the rearing tanks should follow ambient laboratory temperatures of 20-25ºC, but sudden, extreme, variations in temperature must be avoided.

8.3.2 When larval and other small-sized fish are acquired from a commercial source, they should be isolated for a short time before use to acclimate to test conditions (see below), and to ensure they are healthy and disease-free. Unhealthy or diseased fish should not be used. In general, if greater than 20% of the fish die within the holding and acclimation period, the fish should not be used.

8.3.3 Acclimation. Like embryos, larval and other small-sized fish should be standing in dilution water within 3ºC of the desired test temperature. To acclimate, fish should not be subjected to more than a 3ºC change in water temperature in any one 12-h period, and preferably not more than 3ºC in 72 hr (ASTM, 2013). The concentration of dissolved oxygen should be maintained between 60 and 100% saturation. To prevent unnecessary osmotic stress, however, it is recommended that while larval and other small-sized fish should not be subjected to more than a 50 mg/L (as $CaCO_3$) change in water hardness in any one 24-h period; acclimation to more than a 100 mg/L in total hardness is acceptable.

8.3.4 Handling. Larval and other small-sized fish should also be handled as little as possible. When handling is necessary, it must be done gently, carefully and quickly so that the organisms are not unnecessarily stressed. In general, small dip nets are best for handling fish that weigh over 0.5g each (for any size less than that use a smooth-bore pipette). Equipment used to handle fish must be sterilized (soap and hot water and well rinsed at a minimum), and hands should be washed before and after handling.

IX. QUALITY CONTROL

9.1 A reliable quality control usually requires 20-30 eggs or fish placed under a microscope or viewed at the macroscopic scale using the same process but analyzed separately by a second analyst immediately following counting by the first analyst, to guard against delayed effects of sample processing.

9.2 The number of quality control samples is project-specific, but should generally reflect a 10-20% effort, and increased based on the number and frequency of errors detected. Note that for small fish, the 10-20% quality control sample effort could amount to re-examination of the appropriate number of fish set aside in a separate container to be analyzed by a second analyst.

9.3 Any deviations from quality control procedures must be recorded – see under Deviations below.

9.4 Chain-of-custody forms must be completed in cases where samples change hands from one party to another, or to a third party. Completion of chain of custody forms as per GLEC SOP LAB 1014.

X. CALIBRATION

10.1 Calibrate meters and probes in the morning (or before use) and again mid-day, or about every three hours when in use, and whenever the meter produces erratic results.

10.2 Follow calibration and maintenance procedures in the parameter/equipment specific SOP.

XI. PROCEDURE

11.1 Microscope and Microscope Use (General)

11.1.1 Microscopes. Depending on the size of the specimen, appropriate microscopes are a reflected-light (dissecting) stereo microscope. For large specimens, a simple table top magnifier-illuminator unit may be acceptable.

11.1.2 General Microscope Use. Ensure the microscope is located on a flat and sturdy surface. Turn on the illumination source (if applicable) and place the slide or other device holding the specimen (watch glass or petri dish) on the stage. The proper position for the sample is directly over the light source. Set the microscope to the lowest objective powered lens and use the coarse and fine focus knobs to bring the image into focus. Always look into the ocular lens with both eyes open, resisting the urge to close one eye and squint with the other which will increase the strain and tension on your eyes. Adjust the diaphragm as needed (and as applicable) to allow more or reduce the amount of light let in. Switch to higher magnification powers as needed, refocusing each time if needed. Wipe the lenses with lens paper if dirty then cover the microscope once finished.

11.2 Microscopic Viability Observations and Viability Analysis of Embryos

11.2.1 The procedure to observe the embryos for viability consists of dividing them in sub-samples, taking each of them out of the exposure or other temporary container, and examining them under a microscope for viability status and/or other irregularities. Eggs can be preserved in ethanol for archive and for obtaining total counts of eggs observed, or, returned to the exposure tank or other temporary container.

11.2.1.1 With a large, smooth-bore pipette, carefully, gently and quickly transfer embryos from the temporary container to a watch-glass or Petri dish, making sure that the embryos form a single layer.

11.2.1.2 Adjust the microscope as described above.

11.2.1.3 Check for the following embryo viability characteristics, depending on the scope of the work:

- At a minimum (and assuming all eggs are fertilized), look for:
    - Presence/absence of heartbeat and/or larval movement and/or opaque discoloration in species whose embryos are normally translucent (depending on stage of development); and
    - Movement after gentle prodding with a pipette.
- On a project-specific basis, look for irregularities such as:
    - irregular rounded shape and size (diameter in fathead minnow approximately 1.2 to 1.6 mm);
    - irregular yolk formation (e.g., a decrease in the amount of vitellogenic/yolk material that is deposited in the developing oocyte); and
    - irregular transparency (no superficial spots and dark areas).

11.2.1.4 Record observations. If the embryo is so undeveloped such that live/dead status cannot be ascertained (via gentle prodding with fine forceps or blunt probe to elicit a response, when necessary), the viability status of the embryo should be recorded as “indeterminate.”

Note: An early estimate of irregular or aborted eggs can be made at this stage. Any irregular egg will soon develop into an abortive embryo or an abnormal larva. Spots on the external chorion account for physical or bacterial damages.

As a general rule, good egg batches have usually less than 10% abnormal eggs. Any batch containing more than 20% abnormal eggs should be discarded or is sign of adverse effect/impact.

11.2.1.5 Sub-samples are taken by gently swirling containers to re-suspend any eggs that have settled to the bottom and using a large, smooth-bore pipette to place a volume (e.g., 10 – 20 milliliters or mL) into a counting chamber (watch glass or petri dish). Volume of sub-sample is driven by number of eggs in sub-sample volume, and should generally not exceed more than 1 egg per 2 mLs.

11.2.1.6 The sub-sampling process is repeated as many times as needed over a specified period of time in order to ensure a certain percentage of embryos is examined within a specified time frame. For example, repeating the sub-sampling process using a 20 mL sub-sample volume from a 1000 mL container 25 times in one hour allows for one technician to evaluate half (500 mLs) the volume of the 1 L container containing embryos. If the estimated number of embryos in the 1 L container is 200 eggs, that researcher will examine approximately 100 eggs in the time allotted.

11.2.1.7 The sub-sampling process, the amount sub-sampled, and the timeframe for sampling is project-specific, but should generally not exceed 100 - 130 eggs per researcher per hour, pending experience.

11.2.1.8 Surviving embryos are returned to exposure tanks/containers, euthanized, or, can be preserved for later analysis.

11.2.1.9 Uncontaminated dead embryos are bagged and discarded according to laboratory specifications, or stored frozen for later analysis and disposal.

11.3 Microscopic and Macroscopic Observations and Viability Analysis of Larval and Small Fish

11.3.1 The procedure to observe larvae or small-sized fish for viability consists of visually verifying that each individual in the test, whether in an exposure treatment replicate of an aquatic toxicity test or in a different container representing a unique treatment/test group from a special study, is viable and/or otherwise free of physical abnormalities or irregularities in appearance. Fish can be preserved in ethanol for archive and obtaining total counts observed, or, returned to the exposure tank or other temporary container.

11.3.1.1 With a small dip net (for fish weighing 0.5 g or greater) carefully, gently and quickly transfer the fish from the temporary container to an appropriately-sized vessel from which to make microscopic or macroscopic observations, depending on the size of the fish.

11.3.1.2 Adjust (dissecting) microscope as described above if it is necessary to use a microscope, i.e., for very small newly-hatched larvae, or, place vessel holding specimen(s) on illuminator box and use visual inspection or inspection with magnifying glass, as needed. The vessel used for inspection can contain one or more fish, so long as the research technician making the observations can distinguish amongst individuals for accurate viability counts. Note, it is not recommended that more than 10 fish be evaluated at any one time in any vessel, and it is preferable to limit the maximum number of fish to 5 at any one time.

11.3.1.3 Check for the following fish viability characteristics, depending on the scope of the work:

- At a minimum (and assuming all fish are intact):
    - Presence/absence of voluntary body movement and/or respiratory (gill) movement and/or heartbeat and/or presence/absence of movement to stimulus (gentle prodding with blunt probe/instrument).
- And, on a project-specific basis, other physical and behavioral irregularities/abnormalities:
    - Uncoordinated swimming and/or skin damage or abrasion (indication of physical damage);
    - Skin damage or abrasion and/or bleeding (indication of physical damage);
    - Improper pigmentation (physical or other damage); and

  - Hyperventilation or surfacing (could be a severe stress response due to handling and not treatment, but could be due to internal damage).

11.3.2 Record observations. Note that in the case of small fish entrainment and other special studies, whole, intact fish may not be available for live/dead counting. In such a case, the fish heads of dead animals can be counted. Additionally, injured/damaged fish, while alive, can be counted separately for viability analysis (presumably, per the definition of viability, injury or damage precludes "ability to survive.").

11.3.3 Sub-sampling for fish is generally constrained by how many fish can be observed accurately at one time, as per above. For most studies, and unlike embryo analysis, all fish tested/treated are assessed for viability, unless sample numbers are excessive for number of technicians to process (usually a limitation in the field, not in the laboratory or via a bench-scale test).

11.3.4 The sub-sampling process is repeated as many times as needed over a specified period of time in order to ensure all (or a certain percentage) of fish are examined within a specified time frame.

11.3.5 The sub-sampling process, the amount sub-sampled, and the timeframe for sampling is project specific.

11.3.6 Surviving fish are returned to exposure tanks/containers, euthanized, or, can be preserved for later analysis.

11.3.7 Uncontaminated dead animals are bagged and discarded according to laboratory specifications, or stored frozen for later analysis and disposal.

XII. DATA ANALYSIS AND CALCULATIONS

12.1 Data analysis and calculations will be project specific. Any marked activities for the project (Project Name, Date, Scope of Work, Location, Sample Type, Sample Numbers, etc.) and any notes or calculations taken/made during the event will be documented in a project-specific log book.

12.2 Dedicated raw data sheets will be generated prior to the study/analysis, and data recorded by hand in hardcopy. Raw data in hardcopy form can be transferred to electronic spreadsheet form, as needed. A 100% QC of data transferred to hardcopy is required. Alternatively, data can be hand-entered in electronic form on-site using a portable computer, but values must be recorded in hardcopy on raw data sheets to ensure transparency and **for QA/QC purposes.**

12.3 Other forms of data reporting are project-specific, and are subject to requests and agreement by the project manager/leader prior to project initiation.

XIII. INSTRUMENT MAINTENANCE

13.1 Maintain equipment and meters and thermometers according to the parameter/instrument specific SOP.

13.2 Cover microscopes when not in use, and replace light bulbs as needed.

XIV. QUALITY ASSURANCE

14.1 Each part of the raw data, data transcription, and final report is reviewed by the primary generator of the information, and then a 100% QC is conducted by a peer familiar with the test. These reviews consist of checking any mathematical computations performed, and the accuracy and traceability of the data, sample IDs and completeness/clarity of the data sheets. The final report is then reviewed by an upper level staff member or a management designee for scientific soundness and assessment of any usual results.

XV. WASTE MANAGEMENT/POLLUTION PREVENTION

15.1 This method will be conducted with active pollution prevention as an objective by: modifying processes to reduce or eliminate waste, promoting the use of non-toxic or less-toxic substances, implementing conservation techniques, and re-using materials rather than putting them into the waste stream.

15.2 Pour liquid waste from this procedure (ethanol) down a sink drain, connected to a municipal sewer system, with the cold water faucet fully open. Allow the faucet water to flow for ~2 minutes.

XVI. DEVIATIONS

16.1 Any change in protocol from an approved study plan must be signed off by the GLEC Quality Assurance Officer after notifying and gaining approval from the client.

16.2 All deviations must be recorded in the project-specific log book, and hardcopies of specific deviations should be provided to the GLEC QAO for record keeping purposes.

16.3 Acclimation of embryos and fish. The recommended magnitude and time rate of change associated with acclimation of embryos and fish noted above are specific to use of organisms for aquatic toxicity testing purposes. In special studies, both magnitude and time rate of change may be altered for specific purposes, but should not exceed thermal or osmotic maxima based on species or taxa-specific guidance, if such guidance exists. Any deviations from the recommended magnitude and time rate of change must be noted in the project-specific log book along with the justification for the deviation.

XVII. REFERENCES (NEEDS COMPLETED AS NECESSARY)

17.1 ASTM E1241-05(2013). Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. ASTM, Volume 11.06.

17.2 Gast, M.H. and Brungs, W.A. 1973. A Procedure for Separating Eggs of the Fathead Minnow. Prog. Fish. Cult. 35:54.

17.3 GLEC SOP LAB 1014. Chain of Custody.

17.4 GLEC SOP TOX 0006. Culturing *Pimephales promelas*.

17.5 Mount, D.I. 1968. Chronic toxicity of copper to fathead minnows (*Pimephales promelas* Rafinesque). Water Research. 2:214-223.

17.6 U.S. Environmental Protection Agency (USEPA). 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Edition. October 2002. EPA-821-R-02-012. USEPA Office of Water, Washington, DC. Appendix A.

Appendix B:
FISH AND FISH EGG STOCKING PERMITS

State of Wisconsin
Department of Natural Resources
Box 7921
Madison, WI 53707

Fish Stocking Permit

Form 9400-61 Rev. 2-01

TO WHOM IT MAY CONCERN

This is to certify that the below named is hereby permitted pursuant to Section 29.745 of the Wisconsin Statutes to import into the state and/or stock fish in the waters specified below. Personally identifiable information found on this form is not intended to be used for any other purpose.

Permittee:

Mark Briggs
3400 Jack Morris Drive
West Branch, MI 48661

989 345 7595

Permit ID# 6812

Permit Issue Date: 07/29/2014

Application Modifications:

Organization: Eastern Reseach Group, Inc.

Stocking Site

County:	Waterbody Name	WBIC:
DOUGLAS	LAKE SUPERIOR	2751220

Status of Water

Public Waters - Public Access

Species to be Stocked

Fish Farm Information	Species	Age	Avg. Length (Inches)	Number	Pounds
Urbank Live Bait, Reg. No.: LIS #35	FATHEAD MINNOW	ADULT	2	1,500	1

To be Obtained From

Supplier Name: HAYWARD BAIT AND TACKLE

THIS PERMIT IS ISSUED SUBJECT TO THE FOLLOWING CONDITIONS:

1. This permit is effective from 08/18/2014 until 10/18/2014.
2. A Receipt of Fish for Planting, Form 3600-16, for all fish stocked in public waters is attached to this Permit. The Receipt must be completed and returned to the Fish Biologist listed on this Permit and Receipt when stocking is complete.
3. Paul Piszczek, telephone number (715) Ur392-7990 , must be notified of the time of stocking at least 3 days in advance of stocking
4. The fish being stocked under this permit must have been certified by a qualified inspector, and must meet fish health standards and requirements promulgated under Wisconsin Stats. 95.60(4s)(b) and Wisconsin Administrative Code s. ATCP Ch. 10.60.
5. A valid signed DATCP Fish Health Certificate (FHC) must accompany the stocking permit during all stocking operations.
6. To prevent or control the spread of the Viral Hemorrhagic Septicemia (VHS) virus, fish may not be moved or stocked into non-VHS infected waters from VHS infected waters, including fish farms that are directly connected to any VHS infected water. VHS infected waters are defined to be Lake Michigan, Lake Superior, Lake Winnebago System and their tributaries upstream to the first dam or barrier impassable to fish.
7. The department reserves the right to inspect all loads of fish to be stocked under this permit, including but not limited to: verify species, count, measure, weigh, assess water quality, or collect tissue samples
8. No fish may be stocked under the authority of this permit if the fish have been transported to their final destination for stocking in any container which also contains any fish not authorized for stocking under this permit. Other fish may be transported on the same truck as long as they are in separate compartments

Biologist Signature: Paul Piszczek (e-signature) Date Signed: 07/29/2014

Return Completed Stocking Receipt to:

Paul Piszczek
Wisconsin Department of Natural Resources
1701 N 4TH ST
Superior, WI 54880

AH-AQ-2002 (rev. 12/2008)

Wisconsin Department of Agriculture,
Trade and Consumer Protection
Division of Animal Health
P O Box 8911
Madison, WI 53708-8911
Phone: 608-224-4887 Fax: 608-224-4871

OFFICE USE ONLY
Import Permit Number: 35MK081914
Date: 8-18-14 10-31-14
Veterinarian's Signature Expiration Date:
DNR Permit Required? Yes or No

FISH IMPORT PERMIT APPLICATION

ATCP 10.62, Wis. Adm. Code, and Sec. 95.60 (Wis. Stats.)

INSTRUCTIONS: Complete all fields, sign, date, and make a copy for your records. If paying with check or money order, submit fees (payable to WI DATCP), the application and copies of the **FISH HEALTH CERTIFICATE** to the address listed above. Legible fax copies are welcome. A copy of the issued import permit will be faxed, and the original will be mailed. Upon receipt of the approved import permit, forward a copy to the fish source. Ensure that the hauler receives copies of the import permit and fish health certificate because they must accompany each shipment into Wisconsin.

I. IMPORTER INFORMATION

(Person/Business that owns fish/fish eggs when the shipment enters Wisconsin.)

Fish Farm Registration Number: (and) Livestock Premises Code:	(or) WI DNR Stocking Permit Number: 6816
Legal Name Eastern Research Group	Contact Name Mark Briggs
Mailing Address 3400 Jack Morris DR	City / State / Zip West Branch, MI 48661
Telephone Number 989 345 2595	Facsimile Number
Stocking Location (if different than mailing address) including City / State / Zip 14 Marine Drive, Superior WI 54880	
Applicant Signature	Date of Application 8-18-2014

II. FISH SOURCE INFORMATION (Fish/fish egg owner outside Wisconsin)

Legal Name US EPA	Contact Name Frank Whiteman
Business Address 6201 Congdon Blvd.	City / State / Zip Duluth, MN
Telephone Number 218-529-5000	Facsimile Number
Location of Fish (if other than business address) including City / State / Zip	Out of State Registration or License Number (and) Livestock Premises Code (if any)

REQUIRED: *Attach a copy of the Fish Health Certificate (FHC) and all laboratory tests that cover the fish/fish eggs for this application. The import permit cannot be processed until the original FHC is accepted at the Division of Animal Health.*

III. PAYMENT METHOD [$90.00 fee per ATCP 10.62(4)(c)]

Payment Method ☐ Check ☐ Money Order ☐ Visa ☒ Master Card	
(To maintain confidentiality, section III (Payment Method) will be shredded after collection of fees.)	
Credit Card Number 5437 0306 8200 0459	Expiration Date (mm/yy) 08/15
Card Holder Address 3400 Jack Morris Drive	City / State / Zip West Branch, MI 48661
Card Holder Signature & Printed Name Mark Briggs	E-mail address (for receipt) Mark.briggs@erg.com

IV. SPECIES INFORMATION

Species Name Fathead Minnow	☐ Fish or ☒ Eggs	Size
Species Name	☐ Fish or ☐ Eggs	Size
Species Name	☐ Fish or ☐ Eggs	Size
Species Name	☐ Fish or ☐ Eggs	Size
Species Name	☐ Fish or ☐ Eggs	Size
Species Name	☐ Fish or ☐ Eggs	Size

V. RECIPIENT INFORMATION (If other than Importer in Section I)

For multiple recipients, copy this page and complete this section for each recipient.

Recipient Number One

Fish Farm Registration Number: (and) Livestock Premises Code:	(or) WI DNR Stocking Permit Number: (or) WI DNR Bait Dealer License Number:
Legal Name	Contact Name
Mailing Address	City / State / Zip
Telephone Number	Receiving Location (if different than mailing address)

Recipient Number Two

Fish Farm Registration Number: (and) Livestock Premises Code:	(or) WI DNR Stocking Permit Number: (or) WI DNR Bait Dealer License Number:
Legal Name	Contact Name
Mailing Address	City / State / Zip
Telephone Number	Receiving Location (if different than mailing address)

VI. HAULER INFORMATION

Legal Name Eastern Research Group	Registration or License Number
Contact Name Mark Briggs	Mailing Address 3400 Jack Morris Dr.
Telephone Number 989 345 9595	City / State / Zip West Branch, MI 48661

The department shall grant or deny an application under ATCP 10.62 within 30 days after receipt of a complete application. If the department denies the application, the department shall issue the denial notice in writing and shall state the reasons for the denial.

Personal information you provide may be used for purposes other than that for which it was originally collected – sec.15.04(1)(m), Wis. Stats. However, any information that identifies the type or number of fish or fish eggs, the supplier, or purchaser will be kept CONFIDENTIAL by the department as required by law [s. 95.60(7), Wis. Stats.]

SUBMIT ORIGINAL WITHIN SEVEN DAYS AFTER ISSUE TO:
Wisconsin Department of Agriculture,
Trade and Consumer Protection
Division of Animal Health
PO Box 8911, Madison, WI 53708-8911
Phone: 608-224-4872 Fax: 608-224-4871

OFFICE USE ONLY

FISH HEALTH CERTIFICATE

ATCP 10.65, Wis. Adm. Code

FISH SOURCE INFORMATION

See Page 2 for required certificate contents based on movement and fish type.

INSPECTION DATE 7/23/14	ISSUE DATE 7/23/14 (A fish health certificate CANNOT be issued until test results are obtained.)
FISH FARM REGISTRATION	LIVESTOCK PREMISES CODE (if any)
FISH FARM NAME Mid-Continent Ecology Div.	OWNER / MANAGER NAME Frank Whiteman
FISH FARM ADDRESS / CITY / STATE / ZIP 6201 Congden Blvd. Duluth, MN 55804	BUSINESS ADDRESS / CITY / STATE / ZIP same
TYPE OF WATER SUPPLY (Check all that apply) ☒ Lake ☐ Stream ☐ Spring ☐ Well ☐ Other (specify) _____	TYPE OF FISH HEALTH CERTIFICATE (FHC) ☐ Annual (Expires 12 months from date issued) ☒ Lot (Expires 30 days from date issued)

List fish species present on date of inspection. FHM (also present RBT, zebra danio, Japanese Medaka)

QUALIFIED FISH HEALTH INSPECTOR INFORMATION (4)

PRINTED NAME Jeffrey A. Lukken DVM		CONTACT TELEPHONE 218-639-1328
LICENSE NUMBER 06785	ADDRESS 602 S. Olaf Ave.	CITY / STATE /ZIP Battle Lake, MN 56515

I certify that fish for any required laboratory tests have been sampled and inspected by lot or facility according to the current version of the Inspection Section of the AFS-FHS Blue Book or the OIE Manual and Code. I have also visually inspected a minimum of 60 fish per species (or 100% of the population for populations of 60 fish or less) and certify that the fish have no gross clinical signs of contagious or infectious diseases except as noted on this form. All laboratory test results are summarized in the table below and the laboratory's report is appended to this document.

SIGNATURE OF QUALIFIED FISH HEALTH INSPECTOR (4) & DATE
Jeffrey A Lukken DVM 7/23/14

Comments on visible signs of contagious or infectious disease (5).
none - see below

LABORATORY TESTING (3)

See Page 2 for accepted test methodology. Number sampled (line 1) and results (line 2).

Species (1)	Lot Number	Age (2)	Number in Lot	Sampling Date	Farm (F) Wild (W)	Viral Hemorrhagic Septicemia	Whirling Disease	Other:	Other:	Other:	Other:
All Fish in this facility have been perpetuated in-house for at least 10 years. the only Fish brought in to the Facility have been RBT from Facility in LaCrosse, WI with appropriate testing.											

Attach copies of laboratory test results required (ATCP 10.65).

State of Wisconsin
Department of Natural Resources
Box 7921
Madison, WI 53707

Fish Stocking Permit

Form 9400-61 Rev. 2-01

TO WHOM IT MAY CONCERN

This is to certify that the below named is hereby permitted pursuant to Section 29.745 of the Wisconsin Statutes to import into the state and/or stock fish in the waters specified below. Personally identifiable information found on this form is not intended to be used for any other purpose.

Permittee:

Mark Briggs
3400 Jack Morris Drive
West Branch, MI 48661

989 345 7595

Permit ID# 6816

Permit Issue Date: 07/29/2014

Application Modifications:

Organization: Eastern Research Group, Inc.

Stocking Site

County:	Waterbody Name	WBIC:
DOUGLAS	LAKE SUPERIOR	2751220

Status of Water

Public Waters - Public Access

Species to be Stocked

Fish Farm Information	Species	Age (PP)	Avg. Length (Inches)	Number	Pounds
USEPA Mid Continent Ecological Division	FATHEAD MINNOW	~~FRY~~ EGGS	0.1	2,000	1

To be Obtained From

Supplier Name: MID-CONTINENT ECOLOGY DIVISION - EPA

THIS PERMIT IS ISSUED SUBJECT TO THE FOLLOWING CONDITIONS:

1. This permit is effective from 08/20/2014 until 10/19/2014.
2. A Receipt of Fish for Planting, Form 3600-16, for all fish stocked in public waters is attached to this Permit. The Receipt must be completed and returned to the Fish Biologist listed on this Permit and Receipt when stocking is complete.
3. Paul Piszczek, telephone number (715) 392-7990 , must be notified of the time of stocking at least 3 days in advance of stocking
4. The fish being stocked under this permit must have been certified by a qualified inspector, and must meet fish health standards and requirements promulgated under Wisconsin Stats. 95.60(4s)(b) and Wisconsin Administrative Code s. ATCP Ch. 10.60.
5. A valid signed DATCP Fish Health Certificate (FHC) must accompany the stocking permit during all stocking operations.
6. To prevent or control the spread of the Viral Hemorrhagic Septicemia (VHS) virus, fish may not be moved or stocked into non-VHS infected waters from VHS infected waters, including fish farms that are directly connected to any VHS infected water. VHS infected waters are defined to be Lake Michigan, Lake Superior, Lake Winnebago System and their tributaries upstream to the first dam or barrier impassable to fish.
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8. No fish may be stocked under the authority of this permit if the fish have been transported to their final destination for stocking in any container which also contains any fish not authorized for stocking under this permit. Other fish may be transported on the same truck as long as they are in separate compartments

Biologist Signature: Paul Piszczek (e-signature) Date Signed: 07/29/2014

State of Wisconsin
e-Payment Services

Exit

Confirmation

You must click the "Continue" button below in order to return to the state agency's website.

Please keep a record of your Confirmation Number, or print this page for your records.

Confirmation Number **WISAHE009551968**

Payment Details

Description	WI Animal Health DATCP Animal Health E-payment Services http://www.datcp.state.wi.us/
Payment Amount	$90.00
Payment Date	08/20/2014
Status	PROCESSED

Payment Method

Payer Name	Mark Briggs
Card Number	*0459
Card Type	Master Card
Approval Code	T7380B
Confirmation Email	Carol.Pauls@wi.gov

Billing Address

Address 1	3400 Jack Morris Dr.
City	West Branch,
State	MI
Zip Code	48661

Appendix C:
PHOTOGRAPHS

Appendix C: Photographs

Day 1: Fathead Minnow Test


Photograph 1: Control & Gravity Nets


Photograph 2: Setting Up Gravity Net


Photograph 3: Setting Up Control Net


Photograph 4: Control Net

Photograph 5: View of Dock (left)

Photograph 6: View of Dock (right)

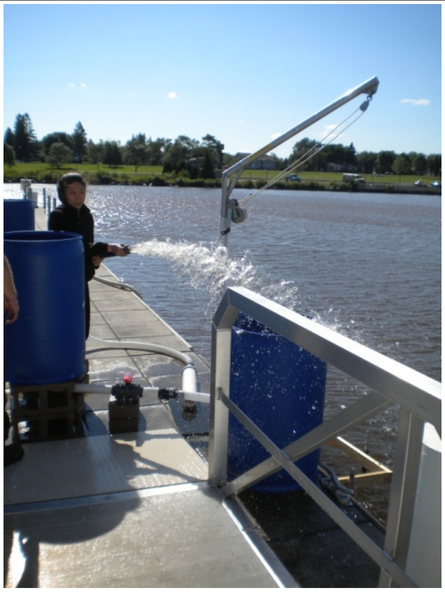
Photograph 7: Net Set-Up

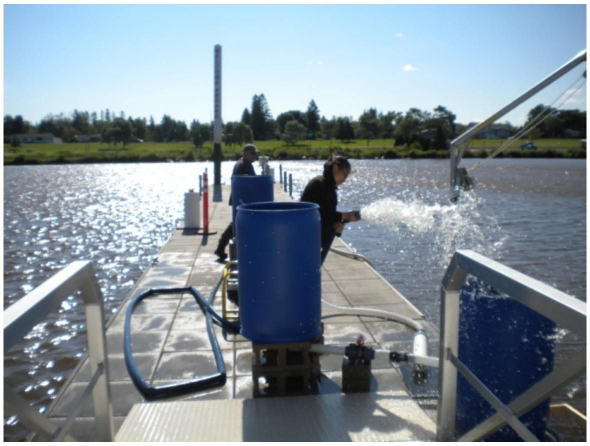
Photograph 8: Security Gate

Photograph 9: Effluent Fish Tanks

Photograph 10: Empty Dock


Photograph 11: Equipment Set-Up Area


Photograph 12: Filling Drums


Photograph 13: Filling Drums 2


Photograph 14: Fish Tank Set-up


Photograph 15: Fish Tank Set-up


Photograph 16: Control Net on Pulley

Photograph 17: GLEC Analysis Area

Photograph 18: Gravity Fish Tank

Photograph 19: Influent Fish Tanks

Photograph 20: Influent Tanks with Aerators and Thermometers

Photograph 21: Influent Fish Tank

Photograph 22: Influent Fish Tanks

Photograph 23: Inside View of Drum

Photograph 24: Indoor Set-up

Photograph 25: NERR Building

Photograph 26: Net Set-up With Hoses

Photograph 27: Net Set-up With Hoses

Photograph 28: Net Set-up

Photograph 29: Pulling the Gravity Net

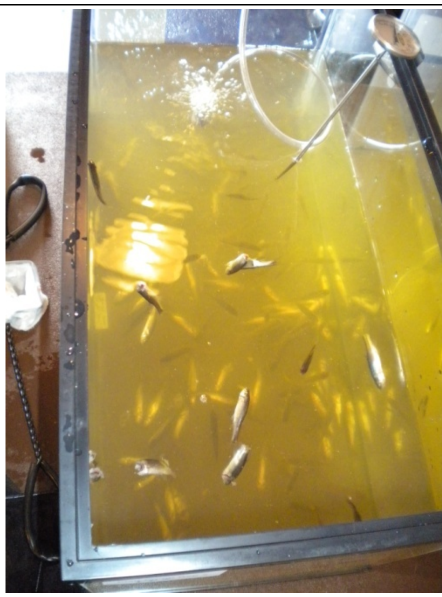
Photograph 30: Pulling the Gravity Net

Photograph 31: Pulling the Control Net

Photograph 32: Pulling the Control Net

Photograph 33: Pulling the Pump Net

Photograph 34: Pulling the Pump Net



Photograph 35: Pump Effluent Replicate 1

Photograph 36: Pump Effluent Replicate 1

Photograph 37: Pump Effluent Replicate 1

Photograph 38: Pump Efflent Replicate 2

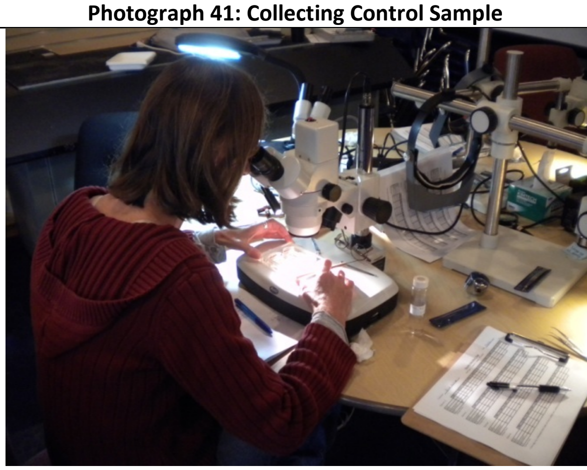
Photograph 39: Pump Effluent in Netting

Photograph 40: Pump Effluent in Netting

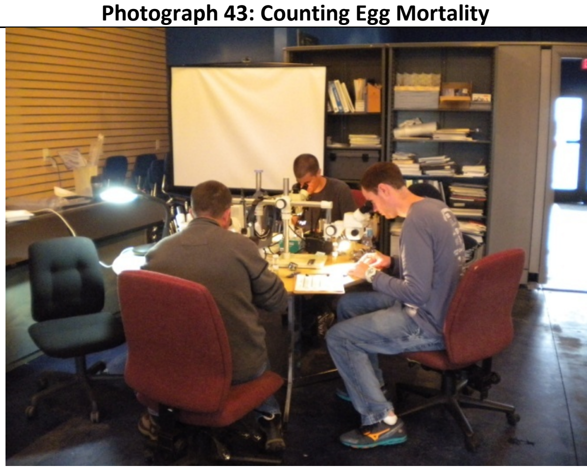
Day 2: Fathead Minnow Eggs Test


Photograph 41: Collecting Control Sample


Photograph 42: Collecting Gravity Sample


Photograph 43: Counting Egg Mortality


Photograph 44: Counting Egg Mortality


Photograph 45: GLEC Analysis Area


Photograph 46: GLEC Analysis Area


Photograph 47: Dock Set-up

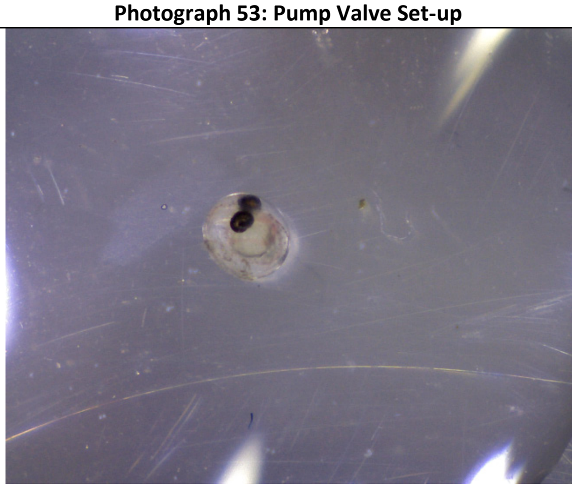

Photograph 48: Emptying Drum


Photograph 49: Effluet Egg Samples


Photograph 50: Influent Eggs


Photograph 51: Three Sample Collecting Area


Photograph 52: Pump Set-up

 **Photograph 53: Pump Valve Set-up**	 **Photograph 54: Trash Pump**
 **Photograph 55: Fish Egg Under Scope**	

Appendix D:
LABORATORY MORTALITY DATA

Fish/Egg Mortality Tests - GLEC log

Fish/Egg	Treatment	Rep #	Date	Time In	Temp Start	Temp 11:27	Temp 11:50	Temp 12:22	Temp 13:50	Temp __:__	Time Finished	Comments
Eggs	Control	1	9/10/14	10:43	17.0	18.5	17.0				11:52	127 processed
Eggs	Gravity	1	9/10/14	10:54	17.0	17.5	17.0				12:06	100% examined
Eggs	Pump	1	9/10/14	11:00	17.0	17.5	17.0				12:00	126 processed
Eggs	Control	2	9/10/14	12:00	16.5			17.0			13:12	100% examined
Eggs	Gravity	2	9/10/14	12:06	16.5			17.0			13:16	100% examined
Eggs	Pump	2	9/10/14	12:11	16.5			15.5			13:20	100% examined
Eggs	Control	3	9/10/14	13:12	16.5				17.0		14:15	100% examined
Eggs	Gravity	3	9/10/14	13:16	16.5				17.0		14:25	100% examined
Eggs	Pump	3	9/10/14	13:20	16.0				16.0		14:20	100% examined

Technician: David Rosier

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☑ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	5	11	10	1							27
Alive	3	2	9	11							25
Injured*	0	0	0	0							0
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Many fish macerated by pump and killed

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	4	3	4								11
Alive	11	10	11								32
Injured*	0	1	3								4
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 2 - slight hemorrhaging at mouth
Sub 3 - Sever damage to head on one, cut in half, one slightly hemorrhaging

Replicate _4__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	9	8	6	3							26
Alive	1	10	4	0							15
Injured*	0	2	1	0							3
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 2 - Damage to head/eyes
Sub 3 - Small cut on flank

Replicate _5__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub __	Sub __	Sub __	Total
Dead	4	2	6	2	11	3	0				28
Alive	9	8	10	12	4	12	10				65
Injured*	0	1	2	0	1	3	4				11
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes: Sub 2 - Hemmorging on face
Sub 3 - Eyes missing, damage to head
Sub 5 - Dorsal hemmoraging and caudal half missing
Sub 6 and 7 - Hemmorrhaging on dorsal and mouth/head

* Fish which are alive but have a noticeable injury. NOTE, these are a subset of alive and all fish should be tallied as either live or dead.

Technician: Eliot Hoitsma

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☑ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	12									12
Alive	10	1									11
Injured*	5	1									6
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Tail fins chopped off

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	5	4	13	1	6						29
Alive	3	16	5	13	1						38
Injured*	1	2	2	2	0						7
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Tail fin chopped off, bleeding from gills

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	2	6	7	5							20
Alive	9	5	2	10							26
Injured*	0	1	1	1							3
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: missing half of body, bent body

Replicate _5__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	14	8	5	14	3						44
Alive	1	3	11	1	5						21
Injured*	0	1	0	0	0						1
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

Technician: Tracey Ledder

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☑ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	14	8	2	2							26
Alive	3	3	11	3							20
Injured*	1	1	0	0							2
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 1 - cut in half
Sub 2 - cut in half

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	13	7	2								22
Alive	12	4	13								29
Injured*	0	0	0								0
QC?	☑ CT	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _4__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	10	6	2	1							19
Alive	16	5	9	6							36
Injured*	2	1	1	1							5
QC?	☑ CT	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 1 - eye damage Sub 3 - mouth injury
Sub 2 - eye damage Sub 4 - mouth injury

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

Technician: David Rosier

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☐ **Pump** ☑ **Gravity**

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	0	0	0	0	0	0	0	0	0
Alive	13	12	13	13	13	13	13	12	13	6	121
Injured*	1	0	0	0	1	0	0	0	2	0	4
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 1 - Nearly unresponsive, minimal movement
Sub 5 - Injured with bloody mouth
Sub 9 - 2 minor injuries, bloody fins

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

Technician: Eliot Hoitsma

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☐ **Pump** ☑ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0									0
Alive	14	6									20
Injured*	0	0									0
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _4__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	0	0	0	0	0	0	0	0	0
Alive	12	12	11	10	13	17	9	9	11	8	112
Injured*	0	0	0	0	0	0	0	0	0	0	0
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _4__

	Sub 11	Sub 12	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0									0
Alive	7	8									15
Injured*	0	0									0
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a subset of alive and all fish should be tallied as either live or dead.

Technician: Tracey Ledder

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☐ **Control** ☐ **Pump** ☑ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	0				0
Alive	22	23	10	20	8	6	4				93
Injured*	0	0	0	0	0	0	0				0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	1				1
Alive	23	15	16	8	10	13	9				94
Injured*	0	0	0	1	0	0	0				1
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Sub 4 - Bloody lip

Replicate _5__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	0	0	0	0	0	0	0	0	0
Alive	11	17	9	12	16	11	10	14	7	11	118
Injured*	0	0	0	0	0	0	0	0	0	0	0
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _5__

	Sub 11	Sub 12	Sub 13	Sub 14	Sub 15	Sub 16	Sub 17	Sub 18	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	0	0			0
Alive	9	13	12	7	6	11	9	10			77
Injured*	0	0	0	0	0	0	0	0			0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

Technician: David Rosier

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☑ **Control** ☐ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0					0
Alive	10	9	10	9	9	3					50
Injured*	1	0	0	0	0	0					1
QC?	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0						0
Alive	15	14	16	15	10						70
Injured*	0	0	0	0	0						0
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _4__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0					0
Alive	14	14	13	12	11	14					78
Injured*	0	0	0	0	0	0					0
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _5__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0					0
Alive	13	12	12	15	15	11					78
Injured*	0	0	0	0	0	0					0
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

Technician: Eliot Hoitsma

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☑ **Control** ☐ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0						0
Alive	11	9	7	7	7						41
Injured*	0	0	1	0	0						1
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes: Bloody face

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	0				0
Alive	9	8	11	8	8	6	10				60
Injured*	0	0	0	0	0	0	0				0
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0									0
Alive	17	6									23
Injured*	0	0									0
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _5__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0						0
Alive	13	10	11	14	13						61
Injured*	0	0	0	0	0						0
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a subset of alive and all fish should be tallied as either live or dead.

Technician: Tracey Ledder

Mortality Data Sheet - Fish

Date: 9/9/2014

Test Type: ☑ **Control** ☐ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	1	1	0								2
Alive	4	8	12								24
Injured*	0	0	0								0
QC?	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	0	0	0	0	0	0	0	0	0
Alive	16	9	8	11	8	9	8	8	8	7	92
Injured*	0	0	0	0	0	0	0	0	0	0	0
QC?	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _4__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	0	0	0					0
Alive	20	9	11	14	11	11					76
Injured*	0	0	0	0	0	0					0
QC?	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Injured*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* Fish which are alive but have a noticeable injury. NOTE, these are a <u>subset</u> of alive and all fish should be tallied as either live or dead.

ERG Ballast Fish Data

Pump	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	TOTAL
Dead	65	51	31	45	72	**264**
Live	56	67	58	51	86	**318**
TOTAL	121	118	89	96	158	**582**
% Mort	53.7%	43.2%	34.8%	46.9%	45.6%	**45.4%**

Gravity	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	TOTAL
Dead	0	0	1	0	0	**1**
Live	113	121	94	127	195	**650**
TOTAL	113	121	95	127	195	**651**
% Mort	0.0%	0.0%	1.1%	0.0%	0.0%	**0.2%**

Control	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	TOTAL
Dead	2	0	0	0	0	**2**
Live	115	152	93	154	139	**653**
TOTAL	117	152	93	154	139	**655**
% Mort	1.7%	0.0%	0.0%	0.0%	0.0%	**0.3%**

Technician: David Rosier

Mortality Data Sheet - Eggs

Date: 9/10/2014

Test Type: ☐ **Control** ☑ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub __	Sub __	Sub __	Total
Dead	0	0	0	1	0	0	0				1
Alive	10	4	48	32	7	6	17				124
Indeterminate*	0	0	1	0	1	0	2				4
QC?	☐ ____	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	2	0	0	0	0	0	0	0	2
Alive	14	5	15	4	8	3	3	1	1	1	55
Indeterminate*	0	0	4	0	1	0	0	0	0	0	5
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	0	0			0
Alive	16	1	11	2	4	1	2	1			38
Indeterminate*	0	0	2	1	0	0	0	0			3
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Indeterminate*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* If the egg is so undeveloped that it does not have a visible heart, it will be recorded as “indeterminate”

Technician: Eliot Hoitsma

Mortality Data Sheet - Eggs

Date: 9/10/2014

Test Type: ☐ **Control** ☐ **Pump** ☑ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub __	Sub __	Total
Dead	0	0	0	1	0	0	0	0			1
Alive	4	4	7	1	1	3	2	4			26
Indeterminate*	0	0	2	1	0	0	0	0			3
QC?	☑ CT	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub __	Sub __	Total
Dead	0	0	0	0	0	0	0	0			0
Alive	4	1	6	4	1	2	3	4			25
Indeterminate*	0	1	1	0	0	0	0	0			2
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub __	Total
Dead	0	0	0	0	1	0	0	0	0		1
Alive	2	1	12	4	18	4	1	4	10		56
Indeterminate*	0	0	0	1	0	1	0	0	0		2
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate ___

	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead											0
Alive											0
Indeterminate*											0
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes:

* If the egg is so undeveloped that it does not have a visible heart, it will be recorded as “indeterminate”

Technician: Tracey Ledder

Mortality Data Sheet - Eggs

Date: 9/10/2014

Test Type: ☑ **Control** ☐ **Pump** ☐ **Gravity**

Replicate _1__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0	2	0						2
Alive	19	6	60	34	8						127
Indeterminate*	2	2	4	1	0						9
QC?	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _2__

	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Total
Dead	0	0	0	0	0	0	0	0	0	0	0
Alive	2	1	8	1	2	3	2	0	7	0	26
Indeterminate*	0	0	3	0	1	0	0	1	0	1	6
QC?	☐ ____	☐ ____	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _2 (Cont)__

	Sub 11	Sub 12	Sub 13	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0								0
Alive	1	1	12								14
Indeterminate*	0	0	0								0
QC?	☐ ____	☑ CT	☑ CT	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage Notes:

Replicate _3__

	Sub 1	Sub 2	Sub 3	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Sub __	Total
Dead	0	0	0								0
Alive	0	0	1								1
Indeterminate*	1	1	0								2
QC?	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	☐ ____	

Damage notes: *NOTE, NO QC WAS DONE DUE TO THE LOW COUNTS, PROCESSING WAS OVER TOO SOON.*

* If the egg is so undeveloped that it does not have a visible heart, it will be recorded as "indeterminate"

ERG Ballast Egg Data

Pump	Rep 1	Rep 2	Rep 3	TOTAL
Dead	1	2	0	**3**
Live	124	55	38	**217**
TOTAL	125	57	38	**220**
% Mort	0.8%	3.5%	0.0%	**1.4%**

Gravity	Rep 1	Rep 2	Rep 3	TOTAL
Dead	1	0	1	**2**
Live	26	25	56	**107**
TOTAL	27	25	57	**109**
% Mort	3.7%	0.0%	1.8%	**1.8%**

Control	Rep 1	Rep 2	Rep 3	TOTAL
Dead	2	0	0	**2**
Live	127	40	1	**168**
TOTAL	129	40	1	**170**
% Mort	1.6%	0.0%	0.0%	**1.2%**

Mortality QC Data Sheet

QA Analyst: Chris Turner **Date:** 9/9/2014

Technician	Fish/Egg	Treatment	Rep #	Sub #	Dead			Alive		
					Orig.	QC	RPD	Orig.	QC	RPD
Ledder	Fish	Control	1	2	1	1	0.0%	8	8	0.0%
Hoitsma	Fish	Control	1	3	0	0	#DIV/0!	7	7	0.0%
Rosier	Fish	Control	1	4	0	0	#DIV/0!	9	9	0.0%
Hoitsma	Fish	Gravity	1	1	0	0	#DIV/0!	14	14	0.0%
Ledder	Fish	Gravity	1	6	0	0	#DIV/0!	6	6	0.0%
Rosier	Fish	Pump	1	2	11	12	8.7%	2	2	0.0%
Ledder	Fish	Pump	1	2	8	8	0.0%	3	3	0.0%
Hoitsma	Fish	Pump	1	2	12	13	8.0%	1	1	0.0%
Hoitsma	Fish	Control	2	2	0	0	#DIV/0!	8	8	0.0%
Ledder	Fish	Control	2	4	0	0	#DIV/0!	11	11	0.0%
Rosier	Fish	Gravity	2	1	0	0	#DIV/0!	13	13	0.0%
Ledder	Fish	Pump	2	1	13	17	26.7%	12	15	22.2%
Hoitsma	Fish	Pump	2	3	13	14	7.4%	5	4	22.2%
Ledder	Fish	Pump	2	2	7	7	0.0%	4	4	0.0%
Rosier	Fish	Control	3	1	0	0	#DIV/0!	15	15	0.0%
Hoitsma	Fish	Control	3	1	0	0	#DIV/0!	17	17	0.0%
Ledder	Fish	Gravity	3	1	0	0	#DIV/0!	23	22	4.4%
Hoitsma	Fish	Pump	3	1	2	2	0.0%	9	9	0.0%
Rosier	Fish	Pump	3	1	4	4	0.0%	11	11	0.0%
Rosier	Fish	Control	4	1	0	0	#DIV/0!	14	14	0.0%
Hoitsma	Fish	Gravity	4	2	0	0	#DIV/0!	12	11	8.7%
Ledder	Fish	Control	4	4	0	0	#DIV/0!	14	14	0.0%
Rosier	Fish	Pump	4	1	9	9	0.0%	1	1	0.0%
Ledder	Fish	Pump	4	1	10	11	9.5%	16	17	6.1%
Hoitsma	Fish	Gravity	4	12	0	0	#DIV/0!	8	8	0.0%
Ledder	Fish	Pump	4	3	2	2	0.0%	9	9	0.0%
Rosier	Fish	Control	5	3	0	0	#DIV/0!	12	12	0.0%
Hoitsma	Fish	Control	5	3	0	0	#DIV/0!	11	11	0.0%

Accuracy:

Dead	Alive
100%	100%
100%	100%
100%	100%
100%	100%
100%	100%
92%	100%
100%	100%
92%	100%
100%	100%
100%	100%
100%	100%
76%	80%
93%	80%
100%	100%
100%	100%
100%	100%
100%	96%
100%	100%
100%	100%
100%	100%
100%	109%
100%	100%
100%	100%
91%	94%
100%	100%
100%	100%
100%	100%
100%	100%

Mortality QC Data Sheet

QA Analyst: **Chris Turner** **Date:** **9/9/2014**

Technician	Fish/Egg	Treatment	Rep #	Sub #	Dead			Alive		
					Orig.	QC	RPD	Orig.	QC	RPD
Ledder	Fish	Gravity	5	3	0	0	#DIV/0!	9	9	0.0%
Rosier	Fish	Pump	5	1	4	4	0.0%	9	9	0.0%
Hoitsma	Fish	Pump	5	1	14	13	7.4%	1	2	66.7%
Rosier	Fish	Pump	5	6	3	3	0.0%	12	12	0.0%
Ledder	Fish	Gravity	5	16	0	0	#DIV/0!	11	11	0.0%

Accuracy:

Dead	Alive
100%	100%
100%	100%
108%	50%
100%	100%
100%	100%

Accuracy:

Dead counts - Only 1 of 33 QC'd subsamples where accuracy was less than target of 90%

Live counts - Only 1 of 33 QC'd subsamples where accuracy was less than target of 90%; difference is excessively low (50%) in the one instance because of very low sample numbers

Precision:
Dead counts - Only 1 of 33 QC'd subsamples where RPD exceeded target of 10%
Live counts - Only 3 of 33 QC'd subsamples where RPD exceeded 10%; RPD is excessively high (66.7%) in the one instance because of very low sample numbers

No perceived data use limitations

Mortality QC Data Sheet

QA Analyst: Chris Turner **Date:** 9/10/2014

Technician	Fish/Egg	Treatment	Rep #	Sub #	Dead			Alive			Indeterminate		
					Orig.	QC	RPD	Orig.	QC	RPD	Orig.	QC	RPD
Ledder	Eggs	Control	1	1	0	0	#DIV/0!	19	19	0.0%	2	2	0.0%
Hoitsma	Eggs	Gravity	1	1	0	0	#DIV/0!	4	4	0.0%	0	0	#DIV/0!
Hoitsma	Eggs	Gravity	1	3	0	0	#DIV/0!	7	7	0.0%	2	2	0.0%
Rosier	Eggs	Pump	1	4	1	1	0.0%	32	32	0.0%	0	0	#DIV/0!
Ledder	Eggs	Control	2	3	0	0	#DIV/0!	8	8	0.0%	3	3	0.0%
Hoitsma	Eggs	Gravity	2	3	0	0	#DIV/0!	6	6	0.0%	1	1	0.0%
Rosier	Eggs	Pump	2	3	2	1	66.7%	15	15	0.0%	4	5	22.2%
Ledder	Eggs	Control	2	12	0	0	#DIV/0!	1	1	0.0%	0	0	#DIV/0!
Rosier	Eggs	Pump	3	1	0	0	#DIV/0!	16	16	0.0%	0	0	#DIV/0!
Hoitsma	Eggs	Gravity	3	3	0	0	#DIV/0!	12	12	0.0%	0	0	#DIV/0!

Accuracy:

Dead	Alive	Indeterminate
100%	100%	100%
100%	100%	100%
100%	100%	100%
100%	100%	100%
100%	100%	100%
100%	100%	100%
50%	100%	80%
100%	100%	100%
100%	100%	100%
100%	100%	100%

Accuracy:

Dead counts - Only 1 of 10 QC'd subsamples where accuracy was less than 100%; difference is excessively low (50%) in the one instance because of very low sample numbers

Live counts - 0 of 10 QC'd subsamples where accuracy was less than 100%

Indeterminate counts - Only 1 of 10 QC'd subsamples where accuracy was less than 100%; difference is low (80%) in the one instance because of low sample numbers

No perceived data use limitations

Precision:

Dead counts - Only 1 of 10 QC'd subsamples where RPD exceeded target of 10%; RPD is excessively high (66.7%) in the one instance because of very low sample numbers

Live counts - 0 of 10 QC'd subsamples where RPD exceeded target of 10%

Indeterminate counts - Only 1 of 10 QC'd subsamples where RPD exceeded target of 10%; RPD is excessively high (22.2%) in the one instance because of low sample numbers

No perceived data use limitations

Fish/Egg Mortality Tests - GLEC log

Fish/Egg	Treatment	Rep #	Date	Time In	Temp Start	Temp 10:40	Temp 10:51	Temp 11:48	Temp 12:01	Temp 13:10	Time Finished	Comments
Fish	Control	1	9/9/14	10:22	20.0	20.0	20.0				10:45	
Fish	Gravity	1	9/9/14	10:27	20.0	20.0	20.0				10:51	
Fish	Pump	1	9/9/14	10:33	20.0	20.0	20.0				10:51	Preserved Specimens
Fish	Control	2	9/9/14	11:36	20.0			20.0	20.0		11:48	
Fish	Gravity	2	9/9/14	11:41	20.0			20.0	20.5		11:57	Preserved Specimens
Fish	Pump	2	9/9/14	11:46	20.0			20.0	21.0		12:03	
Fish	Control	3	9/9/14	12:48	20.0					20.0	12:58	
Fish	Gravity	3	9/9/14	12:51	20.0					20.0	13:05	Preserved Specimens
Fish	Pump	3	9/9/14	12:54	20.0					20.0	13:05	

Fish/Egg	Treatment	Rep #	Date	Time In	Temp Start	Temp 14:15	Temp 14:23	Temp 15:20	Temp __:__	Temp __:__	Time Finished	Comments
Fish	Control	4	9/9/14	13:58	20.0	20.0	20.0				14:08	
Fish	Gravity	4	9/9/14	14:00	20.0	20.0	20.0				14:16	
Fish	Pump	4	9/9/14	14:04	20.0	20.0	20.0				14:23	Preserved Specimens
Fish	Control	5	9/9/14	15:05	20.0			20.5			15:14	
Fish	Gravity	5	9/9/14	15:06	20.5			20.5			15:30	
Fish	Pump	5	9/9/14	15:08	20.5			20.5			15:33	

Initial Examination of Eggs prior to testing

Live	Dead	Indeterminate	% Mortality	QC'd
5	0	2	0.0%	☑
7	0	1	0.0%	☐
7	4	1	36.4%	☐
7	0	0	0.0%	☑
14	0	1	0.0%	☐
18	1	2	5.3%	☐
8	0	1	0.0%	☐
13	0	0	0.0%	☑
9	0	1	0.0%	☐
88	**5**	**9**	**5.4%**	

Appendix E:
LABORATORY QUALITY ASSURANCE DATA

<u>ERG Superior Ballast Water - Quality Assurance Summary and Statement Regarding Data Use</u>

Primary Data collected by:

GLEC Senior Tech	Dave Rosier
GLEC Tech	Eliot Hoitsma
LSNERR Tech	Tracey Ledder

Field QC Analyst: Chris Turner

QA/QC effort included:

100% QC of raw data against data entered into Excel spreadsheet by Deb Turner (GLEC).

Additional 10 to 20% QA of comparison of raw data against data entered into spreadsheet, including all calculations by Tyler Linton (GLEC).

100% QA of information from chain of custody against data spreadsheet by Tyler Linton (GLEC).

Results:

No errors found in QA effort

Egg Analysis:

<u>Analytical Completeness</u>:

QC goal was at least 1 of every 10 samples checked by a second analyst; also at minimum one of every subsample per replicate checked by a second analyst. Goal was met.

10 of 68 subsamples QC'd = Duplicate QC of every 6.8 subsamples observed; Chris Turner (CT)

<u>Precision:</u>

Dead counts - Only 1 of 10 QC'd subsamples where RPD exceeded target of 10%; RPD is excessively high (66.7%) in the one instance because of very low sample numbers

Live counts - 0 of 10 QC'd subsamples where RPD exceeded target of 10%

Indeterminate counts - Only 1 of 10 QC'd subsamples where RPD exceeded target of 10%; RPD is excessively high (22.2%) in the one instance because of low sample numbers

<u>Accuracy:</u>

Dead counts - Only 1 of 10 QC'd subsamples where accuracy was less than 100%; difference is excessively low (50%) in the one instance because of very low sample numbers

Live counts - 0 of 10 QC'd subsamples where accuracy was less than 100%

Indeterminate counts - Only 1 of 10 QC'd subsamples where accuracy was less than 100%; difference is low (80%) in the one instance because of low sample numbers

Findings –

No perceived data use limitations based on completeness, precision or accuracy

Fish Analysis:

Analytical Completeness:

33 of 163 subsamples QC'd = Duplicate QC of every 4.9 subsamples; Chris Turner (CT)

Precision:

Dead counts - Only 1 of 33 QC'd subsamples where RPD exceeded target of 10%
Live counts - Only 3 of 33 QC'd subsamples where RPD exceeded 10%; RPD is excessively high (66.7%) in the one instance because of very low sample numbers

Accuracy:

Dead counts - Only 1 of 33 QC'd subsamples where accuracy was less than target of 90%
Live counts - Only 1 of 33 QC'd subsamples where accuracy was less than target of 90%; difference is excessively low (50%) in the one instance because of very low sample numbers

Findings –

No perceived data use limitations based on completeness, precision or accuracy