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Report on Vitellogenin Gene Expression in Fathead Minnows and Pearl Dace from Reference (non-dosed) and Lakes Dosed with EE2 in the Canadian Experimental Lakes Area

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Notice

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1 ABSTRACT

A whole-lake endocrine disruption experiment was conducted by Fisheries and Oceans Canada at the Experimental Lakes Area (ELA) in northwestern Ontario for three years beginning in 2001. This experiment examined population, organismal, biochemical and cellular-level effects in lake trout, white sucker, fathead minnow, and pearl dace exposed to environmentally-relevant (4-6 ng/L) concentrations of the synthetic estrogen, 17α-ethynylestradiol. The USEPA collaborated in this study by evaluating vitellogenin (vtg) gene expression in: 1) indigenous fathead minnows and pearl dace collected from the dosed and reference lakes in 2001 through 2003, before and after dosing; 2) indigenous minnows collected in 2001from the reference lake and deployed for 1, 3, 7 and 13 days in the dosed and reference lakes; and 3) Cincinnati cultured minnows exposed to water collected in 2001 through 2004 from ELA lakes and shipped to Cincinnati. RT-PCR methods were used to measure vtg expression. In addition to water exposures, embryo-larval fish and adult male fathead minnows were exposed to reference and dosed lake sediment elutriates. Indigenous male fathead minnows and pearl dace collected at all time intervals from the dosed lake showed a constant level of elevation in vitellogenin gene expression. Gene expression in the 2001 fathead minnow deployment study was detected within 24 hours after deployment of control fish into the treated lake and stayed elevated for the entire 13-day study. Highly variable gene expression was found in fathead minnow fry exposed to dosed lake sediment elutriates, but no significant gene expression was found in fry exposed to reference lake sediment elutriates. Male adult fathead minnows exposed to elutriates from sediments collected in 2004 in the previously dosed lake showed significant vitellogenin gene expression. Results indicate that RT-PCR analyses of total RNA can be used to provide a rapid and timely estimate of exposure to estrogenic substances.

2 INTRODUCTION

There is increasing concern about the potential impact of endocrine-disrupting compounds (EDCs) on aquatic organisms. EDCs are compounds that interfere with the normal functioning of hormones in the body. Among the EDCs that are found in aquatic habitats are synthetic estrogens, which are used in contraceptives and other pharmaceuticals. These chemicals enter waterways through sewage treatment plants or septic systems and are also found in surface waters (Länge et al 2001). One of the most commonly used synthetic estrogens is 17α-ethynylestradiol (EE2). Several studies have found EE2 in waters downstream of sewage treatment plants. In these rivers, concentrations of EE2 in the low ng/l levels are common, with some sites having concentrations as high as 40-60 ng/l (Desbrow et al 1998; Belfroid et al. 1999; Larsson et al. 1999; Ternes et al. 1999).

Often the weakest link in determining whether observed adverse effects in humans and/or wildlife are linked to EDCs is the absence of adequate exposure data. Most exposure information has focused on the presence of persistent organic pollutants in Europe and North America. Data on the magnitude and trends of global human or wildlife exposure are limited. Potential sources of exposure are through contaminated food, contaminated groundwater, combustion sources, and contaminants in consumer products. Information on exposure during critical development periods is generally lacking. The exposure data sets that exist are primarily for various environmental media (air, food, water) rather than the most relevant internal exposure (blood, tissue). Limited exceptions are human breast milk and adipose tissue samples. Worldwide, despite large expenditures of money, time, and effort, comparable data sets for assessing exposures to EDCs for humans or wildlife are not available. Such information is

essential to evaluate exposure–response relationships adequately in field and epidemiology studies and to use these relationships to produce credible risk assessments.

Vitellogenin is an egg yolk protein precursor that is produced by female fish prior to spawning. Its synthesis is initiated in response to rising circulating estrogen levels. Males do not normally produce this protein, but exposure to estrogenic contaminants initiates its production. Plasma vtg levels in males exposed to estrogenic substances can increase several thousand fold, to levels as high as or higher than that found in females (Palace et al. 2002). Although the presence of plasma vtg protein can be used as an indicator of exposure to estrogenic compounds, our laboratory has developed a reverse transcription-PCR method for quantifying vtg gene messenger RNA (Lattier 2002). Because the presence of circulating protein occurs much later than transcription of the vtg gene and is modulated by numerous control mechanisms, quantitation of vtg gene transcription is potentially a more sensitive and immediate indicator of exposure.

In order to investigate the effects of long-term exposure to an endocrine-disrupting compound on a whole lake ecosystem, Fisheries and Oceans Canada dosed a lake in the Experimental Lakes Area (ELA) of northwestern Ontario with 17α-ethynylestradiol (EE2) for three years beginning in 2001. EE2 was added to the lake at environmentally relevant levels with a target concentration of ~4-6 ng/L. The U.S. EPA collaborated in this study by evaluating vitellogenin gene expression in indigenous fathead minnows (*Pimephale promelas*) and pearl dace (*Margariscus margarita*) collected from a reference lake and a continuously dosed lake and in laboratory cultured fathead minnows exposed to water and sediments from the dosed lake and reference lakes. The purpose was to further develop a vitellogenin gene expression assay as an indicator of estrogen exposure.

3 METHODS

3.1 EE2 Additions to Lake 260v

Palace et al (2002) described the procedures for EE2 addition and determination of lake EE2 concentrations. Briefly, EE2 (Schering Pharmaceuticals, Germany) was dissolved in 100% HPLC grade methanol (Caledon Laboratories), diluted with lake water to 50% solution, and released into the propeller wash of a boat as it was driven around the lake. The amount of EE2 that was added was calculated based on the depth of the surface water layer (epilimnion) and the previous week's data for Lake 260 during the open-water season. The target concentration of EE2 in the epilimnion was 5 ng/L. EE2 was added at a rate of about 5% per addition to the lake three times weekly. Replicate 1 L water samples were collected weekly at five sites around the lake. Prior to analysis testosterone was added to each sample as an internal standard. Samples were filtered and eluted (15-20 ml/min) through pre-cleaned and pre-conditioned C-18 SPE cartridges (Supelco). EE2 was eluted from the column using 100% methanol. Samples were dried under pure N_2 and reconstituted in enzyme immunoassay (EIA) buffer. EE2 and testosterone were quantified using radioimmunoassay and EIA plates (Cayman), respectively. Recoveries of the internal standard ranged from 88% to 109%. EE2 concentrations were corrected for weekly blank values but not for the recoveries of the internal standard (Palace et al. 2002).

3.2 Study Site and Fish Collections

Figure 1 shows the general study area and the location of the lake to which EE2 was added (Lake 260), as well as the lakes used as reference systems for the experiment (Lake 114 and Lake 442). Fathead minnows were collected during 2000 through 2004 using live minnow traps set overnight in epilimnetic waters near shore (<3m) in each of the lakes. Traps were

emptied into holding pens containing aerated water from the appropriate lake, and fathead minnows were sorted from the other resident species (Palace et al. 2002). Livers were removed from males and females, placed in RNAlater and stored at 4° C for at least 24 hours. Samples were stored at -20°C throughout the dosing season and then shipped to Cincinnati on ice.

3.3 2001 Field Deployment Study (Study 1-21)

Fathead minnows were collected using minnow traps in reference Lake 114 two days prior to deployment. Males and females were housed together until the day of deployment, at which time the sexes were separated. Only males were used in the deployment study. Males were deployed in cages in reference Lake 114 and dosed Lake 260. The cages were suspended several feet below the water surface and held in place by anchors and buoys. Fish were provided no food during the period of deployment; however, the cages allowed for the free movement of water and suspended materials. Minnows were retrieved from cages on days 1, 3, 7 and 13 of deployment and the livers excised and place in RNAlater. Gel-based RT-PCR was performed on these samples.

3.4 2001–2004 Studies: Description and Identification of Laboratory Exposures

Several studies were performed with fathead minnows (embryo/larvae, fry and adults) exposed to water collected from ELA lakes and to sediment elutriates from these lakes. All exposures were done in an environmental chamber at 25°C. All fish used for laboratory exposures were reared in-house at the USEPA Cincinnati, Ohio aquatic culture unit maintained by SoBran, Inc. Moderately hard reconstituted water (MHRW) (hardness of 100 mg/l as $CaCO_3$) was used as the control or dilution water for all tests. It was prepared from a standard formula using reagent grade chemicals and Super-Q® ultrapure water. The water was prepared three days prior to the start of the test, to allow sufficient time for stabilization.

3.4.1 Studies 1-11, 1-12, and 1-26

3.4.1.1 Lake Water and Sediment Sample Collection

Water samples were collected in polyethylene cubitainers and the sediment samples were collected in polyethylene bags. The samples were shipped on ice in coolers. All arrived at the AWBERC facility in good condition. A subsample of each water column sample was collected and taken to the seventh floor for use in testing. All unused samples were stored at 4° C for use in any renewal tests. The sediment samples were stored at 4°C until they were used.

No dilutions were prepared for these tests; all samples were tested as 100% receiving water samples. The water was transferred directly from the cubitainers to the test beakers. Once the sample or control water was placed into the test beakers, the beakers were covered and placed into a 25°C environmental chamber, to bring the samples to test temperature. For test renewal, the water was warmed up to the correct temperature in the cubitainer. For embryo-larval tests, in addition to the six lake elutriate samples and a 5 ng/l positive control, a MHRW + DMSO solvent blank was also included.

The first set of samples was received in good condition, but the laboratory was contacted by representatives of the ELA to tell us not to use the first set of samples. It was believed they were collected using a sampling container that might have been contaminated with hydrocarbons, i.e., oil and gasoline from an outboard motor. The second set of water samples was received in good condition. Tests with these samples (received 6/22/01) were started on 6/22/01. The two water samples received were from Lake 114, the reference lake, and Lake 260, the lake being dosed with EE2. Sediment samples were also collected during the first sampling event. These samples were collected 6/18/01 and received in Cincinnati on 6/20/01.

3.4.1.2 Elutriate Preparation

Study 1-26: In addition to the water column samples, six sediment samples were received for analysis using the FHM embryo-larval sediment toxicity testing method. These included one sample from Lake 114 and five samples from various locations in Lake 260. The samples consisted of a loose, flock-like material, making it easy for the eggs to sink below the surface. To deal with this problem, the tests were initiated using mesh screens to support the eggs, but this did not work, as any handling of the test containers re-suspended the sediment and covered the eggs. After three days, all eggs exposed on the sediments from both lakes were dead. Survival after three days was good on the control sand (91.88%), MHRW+DMSO (92.50%) and the 5 ng/l EE2 positive control (93.13%). These results indicated mortality was due to the sediment samples, most likely from physiochemical factors. The decision was made to test these samples as a sediment elutriate, to provide some data to determine if the EE2 in Lake 260 was passing into the sediments.

Elutriates were prepared by adding 1L of sediment to 2L of MHRW and mixing on a shaker table for one hour. The 2:1 water to sediment ratio is a deviation from that described by US Army Corps of Engineers. This ratio was used so that any contaminants that leached from the sediment and into the elutriate water would be more concentrated and therefore have a higher probability of having an effect on the fish. After one hour on the shaker table, the liquid phase was separated from the solid phase by centrifuging for 20 minutes at 3000 RPM. The liquid phase (elutriate) was poured off and collected in glass bottles for use in the embryo-larval toxicity test. These tests were started the day after the elutriate samples were prepared.

3.4.1.3 Study Animals

The adult fish used in study **1-11** were 13-month-old males that had been separated from females for four weeks.

The larval fish used in study **1-12** were between 24 and 48 hours old at the start of the exposures. Fish that are 24 to 48 hours old at the start of a test are close to 96 hours old at the end of a 48 hour exposure. The embryos used in study **1-26** were 24 to 48 hours old at the start (so that fertility could be determined). The eggs were treated with 2mg/l methylene blue for 15 minutes to inhibit the growth of fungus. After the treatment, eggs were transferred to fresh water and held overnight. The eggs were removed from the spawning tiles the day of the test, to prevent damage from overnight aeration. Infertile eggs were removed prior to test setup.

3.4.1.4 Fathead Minnow Embryo-Larval Sediment Elutriate Exposures

Study 1-26: The test chambers used for the fry exposure procedure (Table 3) were 125 ml polystyrene cups. The exposure procedure used a 50 ml test solution volume, 40 eggs per replicate test chamber and four replicate test chambers. All tests lasted five days, with a test solution renewal after 24 hours. Table 3 contains a summary of the test conditions.

To start the fry test, 40 FHM eggs (24 to 48 hours old) were counted into each of four replicate test containers. A different pipette was used to add fish to each treatment in order to prevent cross-contamination of the samples. Once all fish were added, the test chambers were covered with individual lids to prevent cross-contamination among the test chambers and placed back into the environmental chamber. Once the test setup was complete, the initial control water and test dilution samples were analyzed for routine chemistries (pH, dissolved oxygen, conductivity, and temperature).

After the first 24 hours of exposure, the water was changed in each sample. Solutions were brought up to 25°C (the test temperature) before use. After first counting and removing any dead eggs/fish in each container, water was changed by pouring off at least 40 ml of the old water and replacing it with fresh test water. An aliquot of water from each replicate test container was composited to analyze for final test chemical parameters. Once the water was changed in all containers, they were placed back into the environmental chamber. This same process was followed for all change days during the test.

3.4.1.5 Fathead Minnow Adult Water Column Exposures

Study 1-11: To start the adult test (Table 2), one adult male fish (from a group isolated from females) was transferred from a container of adult males to the replicate test chamber using a coarse mesh nylon net. The net was rinsed in hot water and then deionized water between uses for each fish, to prevent the contamination of a replicate with DNA from another fish. After the fish were added, the containers were placed into a 25°C environmental chamber. A pipette attached to an air source was also placed into each container, to provide for aeration of the test water. The air was added at the rate of 100 bubbles/minute. The test chambers used for the adult exposure procedure were 4L glass jars, with 3L of test solution in each replicate. The adult test used five replicate test chambers, with one fish/replicate. These tests used a 24-hour duration.

3.4.1.6 Fathead Minnow Larvae (Fry) Water Column Exposure

Study 1-12: The test chambers used for the fry exposure procedure (Table 1) were 500 ml glass beakers. The exposure procedure used a 400 ml test solution volume, 40 fish per replicate test chamber and 4 replicate test chambers. All tests used a 48-hour duration, with a test solution renewal after 24 hours. Table 1 contains a summary of the test conditions.

To start the fry test, 40 FHM larvae (24 to 48 hours old) were counted into each of four replicate test containers. A different pipette was used to add fish to each treatment, as a means to prevent cross-contamination of the samples. Once all fish were added, the test chambers were covered with individual lids, to prevent cross-contamination between the test chambers, and placed back into the environmental chamber. Once the test setup was complete, the initial control water and test dilution samples were analyzed for routine chemistries (pH, dissolved oxygen, conductivity, and temperature).

After the first 24 hours of exposure, the water was changed in each sample. The required control, solvent blank or toxicant concentration was prepared and all prepared samples or collected water samples were brought up to the 25°C test temperature. The water was changed by first counting and removing any dead fish in each, then pouring off at least 200 ml of the old water and replacing it with fresh test water. An aliquot of water from each replicate test container was composited, to analyze for final test chemical parameters. Once the water was changed on all beakers, they were placed back into the environmental chamber.

3.4.1.7 Positive controls

The laboratory control treatment for a test was a MHRW control. In addition, a 5 ng/l EE2 positive control was conducted concurrently with all tests, to provide a measure of the level of gene expression using a known toxicant. A DMSO control blank was not included in the water column test, but a labline water control was, to determine if effects were seen when animals were placed directly into MHRW from the labline. Labline is the AWBERC in-house culture water, prepared by carbon-filtering Cincinnati tap water and adding calcium chloride to supplement the water hardness. The MHRW + DMSO solvent blank was included in the elutriate tests. Preparation of the solvent blank and/or positive control for all tests required the addition of

3.57 µl of DMSO or EE2 stock to each liter prepared. The EE2 was prepared such that the addition of 3.57 µl of stock resulted in a nominal concentration of 5 ng/L EE2.

3.4.2 Studies 1-23 and 1-24

3.4.2.1 Sample collection and exposures

A three-week study was initiated to determine the effects of long-term exposures to EE2 on vitellogenin gene expression levels in adult FHM males. The study was designed so that adult FHM males could be exposed to samples shipped from the Canadian Experimental Lake Area (ELA) over a three-week period, with renewal of the water in the tanks every three to four days. The water was to be changed a total of five times during the period of the test exposure. In addition, both the new and old water samples were tested with FHM larvae (24 to 48 hours old). This provided an estimate of the variation in the gene expression in the larvae each time a new sample was tested and also an estimate of the level of effect still exhibited by the old water samples.

The above description is the plan that was to be implemented. Circumstances prevented that from occurring. The first set of samples was received on 8/2/01. Two coolers, each with two 20L carboys of sample, were received. The samples received were from Lake 114 (the reference lake) and Lake 260 (the lake dosed with EE2). The next set of samples was received on 8/15/01 and used for a water change that day. This was an emergency collection and sample shipment from 8/14/01. Samples were collected and shipped on 8/7/01, but these were not received until 8/16/01. It was decided these were too old to be of any use. A replacement for this set of samples was collected and shipped 8/10/01. These were received on 8/17/01. It was decided these samples would be used for part of the last water changes with the study. So, due

to problems with shipping, the samples collected 8/14/01 were received and used for a water change on 8/15/01. The water left from the 8/14/01samples was then mixed with the water received on 8/17/01 (collected 8/10/10) and used for the 8/18/01 and 8/20/01 water changes.

3.4.2.2 Study Animals

The adult fish used in study **1-23** were FHM males that were 10 to 12 months old at the start of the test. These fish had been isolated from female animals for two months prior to the start of the test.

The larval fish used in study **1-24** were between 24 and 48 hours old at the start of the exposures. The adult fish used were 13-month-old males that had been separated from females for 4 weeks.

3.4.2.3 Fathead Minnow Larvae (Fry) Water Column Exposure

Study 1-24: The first test with the FHM fry was conducted using PVC exposure chambers developed for use in exposing *Hyalella azteca* in field studies. The purpose for this was to expose the fry animals in the same tanks as the adults. These chambers are 6 cm long and 4 cm in diameter. The ends are threaded and accept an open end cap. The ends were closed using Nitex® mesh screen cut to size. Twenty animals were added to each test chamber, with each tank receiving eight test chambers. Tests with these chambers were not successful, due to excessive mortality in all chambers for all samples tested. Due to the mortality problem, a switch was made to a more conventional fry-exposure method.

The test chambers used for the remaining fry exposure procedures (Table 1) were 500 ml glass beakers. The exposure procedure used a 300 ml test solution volume, 40 fish per replicate test chamber and four replicate test chambers. All tests used a 48-hour duration. The tests were

conducted as static, non-renewal, due to the lack of sufficient lake sample for renewals. Table 2 contains a summary of the test conditions.

No dilutions were prepared for these tests; all samples were tested as 100% receiving water samples. To limit the amount of sample transported from the sample cold room, located in room 464, a secondary cubitainer was used to transfer known quantities of sample from the fourth floor to the seventh floor. All samples were transported inside a cooler, to limit the possibility of a spill. The lake water was brought up to the test temperature, by placing the sample cubitainers into the water bath. The control, solvent blank and positive control samples were prepared in room 783. Since the water in the MHRW carboy was at 24°C, no temperature adjustment was necessary. These samples were prepared, then either added to the tank to fill or used for the water renewal.

To start the fry test, 40 FHM larvae (24 to 48 hours old) were counted into each of four replicate test containers. A different pipette was used to add fish to each treatment, as a means to prevent cross-contamination of the samples. Once all fish were added, the test chambers were covered with individual lids to prevent cross-contamination between the test chambers, and placed back into the environmental chamber. Once the test setup was complete, the initial control water and test dilution samples were analyzed for routine chemistries (pH, dissolved oxygen, conductivity, and temperature).

After the first 24 hours of exposure, the water temperatures were checked and any dead fish removed.

3.4.2.4 Fathead Minnow Adult Water Column Exposures

Study 1-23: The adult fish were tested in glass aquaria, with 20L of test solution in each tank. The MHRW control and the DMSO solvent blank tests were conducted in 40L glass aquaria.

The positive control, Lake 114 and Lake 260 tests were conducted in 80L glass aquaria. This was necessary, so that three tanks (2-40L and 1-80L) could be placed into one of the minnow cool tanks being used as a water bath. The second minnow cool water bath held two 80L aquaria. The minnow cools were used as the means to maintain constant temperature. Water at 25°C flowed into these tanks and supplemental heat was provided by electric aquarium tank heaters. A total of 30 fish was initially exposed in each tank, to provide sufficient numbers of fish so that five fish could be sacrificed on days 1 and 4. Feeding was done twice each day, but the feeding was reduced after the water samples failed to arrive. The fish were fed once every two days, until fresh sample was received, at which point the regular feeding regime was resumed. Table 1 contains a summary of the test conditions. The tests were started on 8/2/01; the water was changed on 8/6/01, 8/15/01, 8/18/01 and 8/20/01.

3.4.3 Lake Water and Sediment Sample Collection

Water samples were collected in polyethylene cubitainers and shipped on ice in coolers. The size of the sample containers prevented the use of a sufficient amount of ice to properly chill the samples. The delays in sample receipt also prevented the samples' arriving at a temperature normally considered acceptable. The samples all arrived at temperatures between 18°C and 21°C. Other than the elevated temperatures, the samples arrived at the AWBERC facility in good condition. A subsample of each was collected and taken to the seventh floor for use in testing. All unused samples were stored at 4°C for use in any renewal tests.

In addition to the two lake samples, a 5 ng/l EE2 positive control and a DMSO control blank were included and treated the same as the lake samples. This provided a means to compare the results from the lake samples to a series of known samples exposed under the same conditions.

3.4.4 Studies 2-19, 2-20, 2-25, 2-27, 2-37, 2-40, 2-56, 2-64 and 2-65

3.4.4.1 Sample collection and exposures

Water and sediment samples were collected three times during 2002; in May, prior to dosing with ethynylestradiol (EE2), in June, after the 5 ng/l EE2 had stabilized and finally in October, the final sample collection period. The reference lake for the first two studies was 114. Due to Lake 114 freezing, Lake 239 was used as the reference lake in October.

3.4.4.2 Elutriate Preparation

Studies 2-25, 2-40 and 2-56: The elutriate samples used in the FHM embryo-larval test were prepared using the elutriate methods described by US Army Corps of Engineers. This was done by mixing 500 ml of sediment with 2000 ml of MHRW, then shaking the sediment:water mixture for one hour at 100 RPM. After the mixing was completed, each sample was centrifuged for 20 minutes at 5000 RPM and the elutriate water collected. Elutriate was stored at 4°C and aliquots were removed and warmed to the test temperature (25°C) as needed daily.

3.4.4.3 Study Animals

The adult FHM male and female fish used in studies **2-19, 2-27, 2-56, 2-20, 2-37,** and **2-64** were 10-12 months old. These male and female fish had been maintained together throughout the rearing and holding period.

The FHM embryo used for embryo-larval tests **2-25, 2-40 and 2-65** were 24 to 48-hour-old embryo obtained from the AWBERC FHM culture unit. The eggs were collected the day prior to the start of the test and held at 25°C overnight. The infertile eggs were removed and fertilized eggs were added to the test containers. The larvae used in study **3-37** were between 36

and 48 hours old at the start of the exposures. The larvae used in study **3-43** were between 24 to 48 hours old at the start of the exposures.

3.4.4.4 Fathead Minnow Embryo-Larval Sediment Elutriate Exposures

Studies 2-25, 2-40 and 2-65: The test conditions for the FHM embryo-larval tests are summarized in Table 8. The embryo-larval test was conducted using FHM embryo that were 24 to 48 hours old at the start of the test. The test used five replicate test chambers, each chamber containing 50 ml of test solution. To start the test, 30 FHM embryos were added to each test container. Each embryo was sorted so that only fertilized, viable embryos were added at the start of the test. The water was changed daily by removing at least 60% of the existing water and adding fresh control, solvent blank, positive control or elutriate sample water back to each test container. Routine chemical parameters were determined on the water that was removed daily. This was done by compositing samples from each replicate as the water was changed. All fresh waters used for test renewal were stabilized at the test temperature before use. Duration of the test is determined by the hatching rate of the embryo. The test was allowed to continue until all embryos were hatched for at least 24 hours. After this occurred, the test was terminated.

3.4.4.5 Fathead Minnow Adult Water Column Exposures

Studies 2-19, 2-27 and 2-56: The test conditions are summarized in Table 6. Five of the fish that were used for the initial setup were sacrificed and the livers collected to provide baseline estimates of the gene expression level activity of the fish at the start of the test. The fish were fed tropical fish flake food. Exposures were conducted in 4L glass jars, filled with 2L of test solution. Test duration was 24 hours. The control and each exposure treatment had five replicate chambers, each containing two adult fish. Once the jars were filled with control water

or test solution, they were covered and placed into the 25°C (±1°C) environmental chamber. Once the temperature had stabilized, the fish were added to each replicate and the jar placed back into the environmental chamber. The remaining initial test solution was analyzed for routine chemical parameters (pH, dissolved oxygen, conductivity, temperature).

An airtube was placed into each replicate chamber and air bubbled in at the rate of 100 bubbles/minute. The airtube consisted of a 1 ml glass pipette attached to a section of flexible plastic tubing. All information (number of animals exposed, test temperature, air flow rate, routine initial chemical parameters, etc.) was recorded on the datasheets.

3.4.4.6 Fathead Minnow Adult Sediment Exposures

Studies 2-20, 2-37 and 2-64: The sediment exposures with the adult FHM followed the methods described above for the water column exposures, with the following modifications (Table 7). The sediment toxicity samples were prepared 24 hours prior to the start of the test by adding 1L of sediment to each replicate test chamber. A 2L volume of MHRW was then added as the overlying water. The MHRW was added such that the sediment was thoroughly agitated. It was believed this would provide for the possible release of any contaminants present in the sediment sample. After the water was added, the test containers were placed into a 25°C (±1°C) environmental chamber and allowed to stabilize overnight. The next day, the control, solvent blank and positive control samples were prepared and brought up to temperature. The test was started as described above. The duration for these exposures was 48 hours, to allow sufficient exposure time to the sediment samples. The water was not renewed.

3.4.4.7 Lake Water and Sediment Sample Collection

Samples were collected by Fisheries and Oceans Canada staff onsite at ELA on 5/21/02, 6/18/02 and 10/23/02. The samples were collected in five-gallon plastic cubitainers and shipped in coolers on ice to AWBERC. Due to the difficulty in shipping overnight from ELA to Cincinnati, all samples were received two days after sample collection. The tests with the water column samples were started on the day the samples were received. The tests with the sediment samples were all started within six weeks of sample collection.

3.4.5 Study 3-37

3.4.5.1 Sample collection and exposures

Three samples from ELA were received, one from Lake 114 at the center buoy and two from Lake 260 (surface sediments from the nearshore area and from the center buoy). All samples were received on September 24, 2003, in good condition. Sediment samples were collected in polyethylene bags and shipped to the laboratory on ice in coolers. All arrived at the AWBERC facility in good condition within three days of collection. Once received, the samples were stored at 4°C, until ready for use in the elutriate preparation.

3.4.5.2 Study Animals

The FHM larvae used in study **3-37** were between 36 and 48 hours old at the start of the exposures.

3.4.5.3 Elutriate Preparation

Study 3-37: Elutriates were prepared by adding 1500 ml of sediment to 1500 ml of MHRW and mixing on a shaker table for one hour. The 1:1 water-to-sediment ratio is a deviation from that

described by US Army Corps of Engineers. However, this ratio was used so that any contaminants leached off the sediment and into the elutriate water would be more concentrated and therefore have a higher probability of having an effect on the fish. After one hour on the shaker table, the liquid phase was separated from the solid phase by centrifuging for 20 minutes at 3000 RPM. The liquid phase (elutriate) was poured off and collected in glass bottles for use in the larval toxicity test. These tests were started the day after the elutriate samples were prepared. A DMSO control blank was used as the laboratory control treatment for the test. EE2 was included (1 µl EE2/L MHRW) as a positive control.

3.4.5.4 Fathead Minnow Embryo-Larval Sediment Elutriate Exposures

Study 3-37: The test chambers used for the fry exposure procedure (Table 9) were 500 ml beakers. The exposure procedure used a 300 ml test solution volume, 40 fry per replicate test chamber and five replicate test chambers. All tests used a 48-hour duration period, with a test solution renewal at the 24-hour period. Table 1 contains a summary of the test conditions. At the end of the test, the fish from each replicate for a concentration were transferred from the test container, counted and placed into labeled vials containing RNAlater buffer. These were first stored at 4°C for 24 hours, then stored at -20ºC until analyzed.

3.4.6 Study 3-43

3.4.6.1 Sample collection and exposures

The purpose of this test was to determine if the addition of a carrier solvent (dimethyl sulfoxide; DMSO) had an effect on making EDCs more bioavailable and eliciting more of gene expression response in the organisms exposed, when compared to a standard sediment elutriate preparation without DMSO. As part of the investigation, three different amounts of DMSO were used to see

if a higher amount would prove more efficient in releasing the EDCs. Two samples were received, one from Lake 239 and one from Lake 260. It was not possible to collect samples from Lake 114, the reference lake routinely used, so samples were collected from Lake 239, another of the reference lakes. All samples were collected 10/30/03 and stored at 4°C until shipped from ELA on Monday 11/03/03. The samples were received at AWBERC on 11/05/03 in good condition. Sediment samples were collected in polyethylene bags and shipped to the laboratory on ice in coolers. The samples were taken to Room 464, the sample preparation and storage room. Once received, the samples were stored at 4°C, until ready for use in the elutriate preparation.

3.4.6.2 Study Animals

The FHM larvae used in study **3-43** were between 24 to 48 hours old at the start of the exposures.

3.4.6.3 Elutriate Preparation

Study 3-43: Elutriates were prepared by adding 1000 ml of sediment to 2000 ml of MHRW and mixing on a shaker table for one hour. The 2:1 water to sediment ratio is a deviation from that described by US Army Corps of Engineers. It was used so that any EDCs released from the sediments would be more concentrated. A series of 12 elutriate samples was prepared, using control sand, Lake 239 sediment and Lake 260 sediment. Each sediment was eluted using 0 µl/L DMSO, 1 µl/L DMSO, 50 µl/L DMSO and 500 µl/L DMSO. As stated above, the various levels of DMSO were used to see if increasing the amount of DMSO provided for more efficient release of the EE2 into the mixing water. After one hour on the shaker table, the liquid phase was separated from the solid phase by centrifuging for 20 minutes at 3000 RPM.

The liquid phase (elutriate) was poured off and collected in glass bottles for use in the larval toxicity test. These tests were started the day after the elutriate samples were prepared. A DMSO control blank was used as the laboratory control treatment for the test. EE2 was included (1 µl EE2/L MHRW) as a positive control.

3.4.6.4 Fathead Minnow Embryo-Larval Sediment Elutriate Exposures

Study 3-43: The test chambers used for the fry exposure procedure (Table 10) were 500 ml beakers. The exposure procedure used a 300 ml test solution volume, 40 fry per replicate test chamber and five replicate test chambers. All tests used a 48-hour duration period, with a test solution renewal at the 24-hour period. Table 1 contains a summary of the test conditions. At the end of the test, the fish from each replicate for a concentration were transferred from the test container, counted and placed into labeled vials containing Tri reagent. The vials of buffer with tissue were homogenized; the vials were then stored at -80°C until removed for RNA isolation and gene expression analysis.

3.4.6.5 Positive controls

17α-ethynylestradiol (EE2) was used as the test positive control chemical. The 10 ng/l concentration was prepared by weighing out the EE2 and dissolving it in a calculated amount of DMSO. Once the first stocks were diluted, a 2 µl aliquot was then diluted with 2 ml of DMSO to prepare the concentrated stock, such that 1 µl of stock was added per liter of MHRW to prepare the positive control test concentration.

TABLE 1. Summary Test Conditions for Larval Fathead Minnow Gene Expression Exposure Tests. **Studies 1-12 and 1-24.**

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	48 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	500 ml
Test Solution Volume	400 ml
Renewal of Test Solution	daily
Age of Test Organisms	24-hour to 48-hour-old larvae
No. Organisms/Test Chamber	30
No. Replicate Test Chambers	4
No. Organisms/concentration	150
Feeding Regime	Not fed
Control and/or Dilution Water	Moderately Hard Water/Labline
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 2. Summary Test Conditions for Adult Fathead Minnow Male Gene Expression Exposure Tests. **Study # 1-11**

TEST PARAMETER	CONDITION
Test Type	static
Test Duration	24 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	4 L
Test Solution Volume	3 L
Renewal of Test Solution	None
Age of Test Organisms	11 to 13-month-old males
No. Organisms/Test Chamber	1
No. Replicate Test Chambers	5
No. Organisms/concentration	5
Feeding Regime	Not fed
Control and/or Dilution Water	Moderately Hard Water or MHRW+DMSO
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 3. Summary Test Conditions for Larval Fathead Minnow Gene Expression Exposure Tests, Embryo-Larval Elutriate. **Study #1-26**.

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	5 days
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	125 ml
Test Solution Volume	50 ml
Renewal of Test Solution	daily
Age of Test Organisms	24-hour to 48-hour-old embryo
No. Organisms/Test Chamber	40
No. Replicate Test Chambers	4
No. Organisms/concentration	160
Feeding Regime	Not fed
Control and/or Dilution Water	Moderately Hard Water
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control hatch and survival

TABLE 4. Summary Test Conditions for Long-Term Exposure of Adult Fathead Minnow Males for Gene Expression Exposure Tests. **Study # 1-23**.

TEST PARAMETER	CONDITION
Test Type	static
Test Duration	21 D
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	40L or 80L
Test Solution Volume	20 L
Renewal of Test Solution	Every 3 to 4 days
Age of Test Organisms	11 to 13-month-old males
No. Organisms/Test Chamber	30
No. Replicate Test Chambers	1
No. Organisms/concentration	30
Feeding Regime	Flake food 2X/day
Control and/or Dilution Water	Moderately Hard Water or MHRW+DMSO
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 5. Summary of Test Conditions for Adult Fathead Minnow Gene Expression Exposure Tests with 2 ELA Water Column Samples.

Study: ELA **Study #: 2-19, 2-27, 2-56** Date: 05/23/02, 06/20/02, 10/27/02

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	24 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	4 L
Test Solution Volume	2 L
Renewal of Test Solution	None
Age of Test Organisms	11, 11, 12-month-old males and females
No. Organisms/Test Chamber	2, 1 male, 1 female
No. Replicate Test Chambers	5
No. Organisms/concentration	10
Feeding Regime	Not fed
Aeration	100 bubbles/min
Control and/or Dilution Water	MHRW, MHRW + DMSO
Sample/Test Material	ELA Lakes 114 (239) and 260, EE2 Positive control
Sample Concentration(s)	Lakes-100%, EE2 5 ng/l
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 6. Summary of Test Conditions for Adult Fathead Minnow Gene Expression Exposure Tests with 2 ELA Sediment Samples.

Study: ELA **Study #: 2-20, 2-37, 2-64** Date: 05/29/02, 02/24/02, 12/16/02

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	48 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	4 L
Test Sediment Volume	1 L
Test Overlying Water Volume	2 L
Renewal of Test Solution	None
Age of Test Organisms	10, 11-month-old males and females
No. Organisms/Test Chamber	2, 1 male, 1 female
No. Replicate Test Chambers	5
No. Organisms/concentration	10
Feeding Regime	Not fed
Aeration	100 bubbles/min
Control and/or Dilution Water	MHRW, MHRW + DMSO
Sample/Test Material	ELA Lakes 114 (239) and 260, EE2 Positive control
Sample Concentration(s)	Lakes-100%, EE2 5 ng/l
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 7. Summary of Test Conditions for FHM Embryo/Larval Gene Expression Exposure Tests with Elutriate Samples Prepared from ELA Sediment Samples.

Study: ELA **Study #: 2-25, 2-40, 2-65** Date: 06/13/02, 08/09/02, 12/18/02

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	5 to 7 days, depending on egg hatching
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	150 ml
Test Solution Volume	50 ml
Renewal of Test Solution	Daily
Age of Test Organisms	24 to 48-hour-old embryo
No. Organisms/Test Chamber	30
No. Replicate Test Chambers	5
No. Organisms/concentration	150
Feeding Regime	Not fed
Aeration	None
Control and/or Dilution Water	Moderately Hard Water, MHRW, DMSO
Sample/Test Material	Elutriate from ELA Lakes 114 (239) and 260 and EE2 Positive control
Sample Concentration(s)	Elutriate-100%, EE2 5 ng/l
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 8. Summary of Test Conditions for Conducting Larval Fathead Minnow Gene Expression Exposure Tests with Elutriate Samples. **Study # 3-37.**

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	48 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	500 ml
Test Solution Volume	300 ml
Renewal of Test Solution	daily
Age of Test Organisms	36-hour to 48-hour-old larvae
No. Organisms/Test Chamber	40
No. Replicate Test Chambers	5
No. Organisms/concentration	200
Feeding Regime	Not fed
Control and/or Dilution Water	Moderately Hard Water + DMSO
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

TABLE 9. Summary Test Conditions for Conducting Larval Fathead Minnow Gene Expression Exposure Tests with Elutriate Samples. **Study # 3-43.**

TEST PARAMETER	CONDITION
Test Type	static-renewal
Test Duration	48 hours
Temperature	25°C (±1°C)
Photoperiod	16 hours light: 8 hours dark
Test Chamber Size	500 ml
Test Solution Volume	300 ml
Renewal of Test Solution	daily
Age of Test Organisms	24-hour to 48-hour-old larvae
No. Organisms/Test Chamber	40
No. Replicate Test Chambers	5
No. Organisms/concentration	200
Feeding Regime	Not fed
Control and/or Dilution Water	Moderately Hard Water + DMSO (1, 50 and 500 ug/L)
Endpoint	Based on gene expression data analyzed
Test Acceptability	90% or greater control survival

3.5 RNA Preparation

Adult fish or fry were sacrificed and adult liver tissues (or whole fry) were suspended in RNAlater solution (Ambion Inc., Austin, TX). Tissues were homogenized using either a hand-held homogenizer or a Mixer Mill 200 (Retsch, Germany) with 3mm stainless steel beads (Qiagen).

Two different techniques were used to extract RNA from individual adult male livers or fry. In the first protocol, which was used with the samples from 2001, total RNA was isolated using the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987) followed by DNAse treatment. For the remaining samples, Tri Reagent was used (Molecular Research Center, Cincinnati, OH).

The integrity of the RNA was determined by visual inspection of the 18s and 28s ribosomal bands on a formaldehyde/MOPS gel. The samples were spectrophotometrically quantified and diluted to a concentration of 1 mg/ml for RT-PCR.

3.6 Gel-based analyses

Reverse transcription polymerase chain reaction (RT-PCR) was performed using GeneAmp® RNA PCR reagents (PE Applied Biosystems, Foster City, CA). Gene-specific oligonucleotides were designed from the FHM vtg precursor mRNA sequence (Preziosi 1998) using Oligo Primer Analysis Software® (Rychlik and Rhoads, 1989). The RT-PCR reactions contained an empirically determined volume of 18S Competimer®/universal ribosomal RNA (rRNA) primer mix (Ambion Inc., Austin, TX) in a muliplex reaction. Reactions also contained Advantage-2 DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA.). Secondary PCR reaction mix was added to the 20 μl reverse transcription reaction for a final PCR volume of 50 μl. The secondary master mix contained 21.5 μl of H_2O, 5 μl of 10X Advantage 2 PCR buffer, 1

μl of Advantage 2 DNA polymerase, 150 nM of each Vg oligonucleotide primer, and 0.5 μl of 5:5 (fry) or 7:3 (liver) ratio of competimer/18S oliginucleotide per reaction.

Thermal cycle number for log linear amplification of vitellogenin gene cDNA for fry and adult liver was determined to be 32 and 17 cycles, respectively. The PCR profile used for both adult liver and fry was: 95° for 1 min; 32 or 17 cycles of 95° for 30 s, 64° for 30 s, and 72° for 30 s; and 1 cycle of 94° for 30 s, 64° for 30 s, and 72° for 10 min to complete any unfinished transcripts.

The amplification products were separated electrophoretically on 1.8% agarose gels. Following electrophoresis, gels were stained in 1X TAE containing SYBR® Green I (Molecular Probes, Eugene, OR) and scanned using a FluroImager® 595 system (Molecular Dynamics, Sunnyvale, CA). Relative intensities for both the Vg and 18S ribosomal bands were analyzed with ImageQuant® (Molecular Dynamics) software.

Relative gene expression was expressed as the ratio of pixel density of the vitellogenin band to the sum of the pixel densities of the vitellogenin and 18S bands [Vg/(Vg+18S); Lattier 2001]. Across replicates, averages and standard deviations were calculated using routines available in Lotus 123 (Cambridge, MA, USA) and Microsoft Excel (Redmond, WA, USA).

3.7 Quantitative Real-time PCR

Total RNA (2 μg) was reverse-transcribed in a 20 μl reaction mixture containing PCR II buffer (Perkin Elmer), 5 mM $MgCl_2$, 2.5 mM total dNTP, 2.5 μM random hexamers, 20 U RNase inhibitor and 2.5 U MuLV reverse transcriptase. Prior to addition of the reverse transcriptase, the reactions were heated to 70°C for 5 min followed by a 15-min ramp to 23°C. This insured denaturation of RNA secondary structure and annealing of the RNA to the random hexamers. Reverse transcriptase was added and the reactions incubated at 48°C for 30 min in a GeneAmp

9600 thermal cycler, followed by 5 min at 95°C to inactivate the enzyme. No template reactions and no reverse transcriptase reactions were run with each set of reactions as controls.

Prior to real-time RT-PCR, 80 µl of sterile, DNase-free, RNase-free water was added to each 20 µl cDNA sample. Real-time RT-PCR was performed using DyNAmo PCR mix (Finnzymes). Reactions (25 µl total) consisted of SYBR Green PCR mix, 3 µl diluted cDNA and 20 pmol primer. Real-time RT-PCR was performed on an MJ Opticon. Reactions were run both with vitellogenin primers and 18S primers. Vitellogenin primers and cycling conditions were developed in this laboratory and will be published in a research article within the next year. The 18S primers used were QuantumRNA 18S universal primers (Ambion) were used as internal standards.

4 RESULTS

4.1 Lake 260 EE2 Concentrations

The target concentration of EE2 in Lake 260 was 5 ng/L. In the first year of dosing, the overall mean epilimnetic concentration of EE2 from 30 May to 4 October 2001 was 6.0±2.8 (SD) ng/L with a high of 8.9 ng/L and a low of 4.0 ng/L (Figure 2). In 2002, mean EE2 concentrations (blank corrected) were 5.0 ± 1.8 ng/L from 30 May to 1 October. Over the addition period in 2003, mean EE2 concentrations (blank corrected) were 4.8 ± 1.0 ng/L between 26 May to 8 October and similar to the two previous years.

4.2 Studies Conducted in 2001

In order to determine the kinetics of vitellogenin expression during the initial period of exposure in 2001, male fathead minnows were collected from Lake 260 after seven weeks, nine

weeks and three months of dosing. Male fathead minnows were collected at the same time from reference Lake 114. Gel-based RT-PCR was performed on samples and vitellogenin expression quantified relative to 18s ribosomal RNA expression.

Vitellogenin was induced in males collected from Lake 260 at all time points (Figure 3). Males collected from Lake 114 had little to no vitellogenin mRNA. Vitellogenin expression in males was comparable to that of females collected from Lake 114 on July 25. The level of expression of vitellogenin in male fathead minnows collected from Lake 260 was statistically different from that of males from Lake 114.

The deployment study was originally supposed to last for three weeks, with five fish withdrawn from each lake after 1, 7, 14 and 21 days of deployment. Due to unexpectedly high levels of mortality, the experiment had to be terminated early. There were not enough fish to continue the study in Lake 114 through day 14, so all seven remaining fish were pulled on day 7. The experiment was also terminated early in Lake 260 since only four fish were remaining on day 13. Male fathead minnows exhibited an increase in vitellogenin mRNA levels after only one day of deployment in Lake 260 (Figure 4). Vitellogenin mRNA levels remained high throughout the study to day 13. Response to EE2 by males was variable, with some fish showing high levels of expression and others showing very little expression. The standard deviations for these samples are quite high. Males in Lake 114 showed no significant expression on days 3 and 7 (Figure 4). However, on day 1 there was a single fish that had elevated levels of vitellogenin mRNA. The other four fish showed no Vg expression.

Elutriate from Lake 114 did not induce vitellogenin in fry, whereas elutriates from sediment from the dosed Lake 260 did (Figure 6). Vitellogenin induction was highly variable in

these samples. Unfortunately, it appears that fry did not respond to water with 5 ng/L EE2. This makes it difficult to interpret these results.

4.3 Studies Conducted in 2002

4.3.1 Fathead Minnows

Fathead minnows were collected at various times throughout the year in 2002 from dosed and reference lakes. Vitellogenin gene expression was determined using QPCR.

Males collected from reference lakes 114, 302 and 442 showed no vitellogenin gene expression throughout the year (Figure 7). Males collected from the dosed Lake 260 on May 27 did not express vitellogenin mRNA, but those collected in June, July and November did. Induction of expression was variable among these fish.

Females collected from reference Lake 442 showed elevated vitellogenin expression in May, while vitellogenin expression in females from Lake 114 was high during June (Figure 8). This may represent differences in breeding season between these two lakes. In contrast, females from the dosed lake 260 continued to have high levels of expression into October, well beyond the breeding season for this species.

4.3.2 Pearl Dace

Pearl dace were collected from dosed lake 260 and reference lakes 114 and 442 at various times during May, June and September in 2002. Vitellogenin gene expression was determined using QPCR.

No vitellogenin gene expression was detected in males collected from Lake 114 June or September (Figure 9). Fish exposed to EE2 in Lake 260 showed variable response in gene

expression. Reference Lake 442 had some fish with elevated gene expression: one fish of the two collected on May 14 and one fish of the seven collected on September 24.

Females in Lake 260 did not exhibit an increase in vitellogenin expression relative to females from other lakes (Figure 10). One female from Lake 442 showed an extremely high level of vitellogenin. However, an examination of the data revealed that this was due to an abnormally low 18S value, which suggests that a failed PCR reaction may be skewing the results for this sample.

The vitellogenin primers used for quantitative real time RT-PCR were originally designed for use with fathead minnows, the sequence of the primers having been based on the fathead minnow vitellogenin gene. These primers appeared to work well with pearl dace samples. We sequenced the pearl dace vitellogenin cDNA to determine its similarity to fathead minnow vitellogenin. The two sequences were 89% similar based on a BLAST search of the sequence. Since the sequencing was done in only one direction, more careful sequencing would likely increase the estimate of similarity.

4.4 Studies Conducted in 2003

4.4.1 Fathead Minnows

Male fathead minnows collected from Lake 260 in May of 2003, prior to the third year of dosing, showed elevated levels of vitellogenin expression (Figure 11). Males from lakes 114 and 442 showed no expression. Female fathead minnows collected from lakes 442 and 260 in May of 2003 both showed elevated levels of vitellogenin expression (Figure 12). No female fathead minnows were collected from Lake 114 in May of 2003.

4.4.2 Pearl Dace

Male pearl dace collected from Lake 260 in May 2003 (prior to dosing) exhibited significant vitellogenin gene expression, while fish from reference lakes 114 and 442 had no vitellogenin mRNA (Figure 13). Female pearl dace showed differences in levels of vitellogenin expression between the dosed and reference lakes (Figure 14). Whether this may be due to differences in breeding season among the lakes or a consequence of the EE2 present cannot be determined. Female fathead minnows showed similar patterns of vitellogenin gene expression between reference Lake 442 and dosed Lake 260.

4.5 Studies Conducted in 2004

No dosing of Lake 260 occurred in 2004. Male fathead minnows exposed to water from Lake 260 did not show an increase in vitellogenin gene expression, with levels similar to those from fish exposed to water from Lake 114 and other control males. Female fathead minnows exposed to water from ELA lakes and the control females showed high variability in vitellogenin expression.

Fry exposed to elutriates prepared from sediments collected from Lake 260 in 2004 showed no increase in vitellogenin gene expression; levels were similar to those from fry exposed to elutriates prepared from lake 114 sediment. In this set of exposures, fry responded to 10 ng/L EE2, although variably.

In 2004 male fathead minnows exposed to sediment elutriate from Lake 260 did show increased vitellogenin expression relative to Lake 114 elutriate. Although results from individual fish varied, one male had a vitellogenin mRNA level that was much higher than that of any males exposed to 5 ng/L EE2.

4.6 Summaries for individual studies from 2001 through 2004

4.6.1 Laboratory Exposures

Study 1-11: The results from the FHM adult male gene induction tests with the MHRW control, labline water, 5 ng/l EE2 and both Canadian water column samples showed no toxicity to the fish in any sample. All fish exposed survived and were sacrificed for necropsy and liver collection at the end of the test. Table 12 contains a summary of the animal survival data, as well as all routine chemical parameters.

Study 1-12: The results for the FHM fry gene induction tests with the MHRW control, labline water, 5 ng/l EE2 and both Canadian water column samples showed toxicity to the fish exposed in Lake 114 (survival = 83.1%, CV = 11.3%) when compared to the fish in the MHRW control (survival = 96.9%, CV = 2.5%), Mann-Whitney Rank Sum Test, P=0.029. The survival of the fish in Lake 260 was not statistically different from that in the control. Table 11 contains a summary of the survival and routine chemical data for these samples.

Study 1-23: The results from the adult FHM male long-term exposure tests (Tables 15-19) are unusual, due to the problems encountered in receiving the shipments of fresh lake water for renewal. On 8/13/01, all fish in the DMSO control tank were found dead. No cause was apparent, so any reason given would be speculation. The water in the tank was 7 days old, but the water in the other tanks was also the same age, so this does not explain the mortality. The next problem was toward the end when the Lake 260 fish began to die. This mortality forced the test to be shut down a day early, to make certain at least 5 fish were still alive for the final necropsy. For the remaining three tanks (control, 5 ng/l EE2 and Lake 114), only 5 ng/l EE2 had any mortality, 2 fish died. No fish died in the control or Lake 114 (the site control). On 8/21/01,

the last fish were necropsied, one day ahead of schedule. At this time, all live fish remaining in each tank were sacrificed, necropsied and the liver tissue collected. The tissue samples from all necropsies were stored in labeled tubes of RNAlater, for future RNA isolation and gene expression analysis.

Study 1-24: The only problem encountered in the FHM fry tests (Tables 20-24) were with the fry exposed in the fish tanks in the mesh-covered chambers. As stated previously, survival in this system was poor, with no sample meeting the minimum control survival criteria of 90% or greater. Control survival was 85%, control + DMSO was 84.4%, 5 ng/l EE2 was 65%, Lake 114 was 82.5% and Lake 260 was 85%. The fish from all treatments were collected and placed into labeled tubes of RNAlater buffer for gene expression level analysis. The next test started 8-6-01, using the remainder of the samples received to start the tests. Survival was acceptable in all treatments. The next set of fry tests started when the next fresh sample (collected 8/14/01) was received on 8/15/01. At this point, the decision was made to test both the new water used for renewal and the old water from the tanks for gene expression level effects. This would provide a means to judge what effects (if any) the old water (now over 2 weeks old and in the tanks for 10 days) would have on the fry. The survival in all samples tested, old and new 114, old and new 260, control, control+DMSO and 5 ng/l EE2, was acceptable. The next renewals, and fry tests, were performed 8/18/01, with samples collected 8/10/01 and received 8/17/01. The water left from the 8/15/01 renewals was mixed in as well. For these tests, both new and old 114 and 260 waters were tested, as well as control, control + DMSO and 5 ng/l EE2. Again, survival in all samples was acceptable. The final tests began 8/21/01. These tests included the last batch of new (?) water for both lakes, old lake water from

8/20/01 and 8/21/01, control, control + DMSO and 5 ng/l EE2. Survival was again acceptable across all samples.

At the end of all fry tests, the fish were removed from the test vessels and transferred into labeled tubes containing RNAlater buffer. They were then stored for future RNA isolation and gene expression level analysis. Due to the lack of sufficient sample, all tests were conducted as static, non-renewal. A set of routine chemical parameters (pH, dissolved oxygen, conductivity and temperature) were determined for the new water used to start the test (initial) and this same water after the 48-hour exposure period (final).

Once the tests were completed, the vials containing the fish or liver samples were stored at 4°C for 24 hours, then at -20°C until processed for RNA isolation and gene expression analysis.

Study 1-26: The results from the FHM embryo-larval tests with MHRW control, MHRW + DMSO solvent blank, 5 ng/l EE2 positive control and the 6 elutriate samples were successful, with acceptable survival in the control (97.5%), positive control (92.5%), and solvent blank (94.4%), Table 13. Survival in the sediment elutriate samples ranged from 70.6% (Lake 260 #1) to 94.4% (Lake 260 #2), Table 13. The survival in the Lake 260 #1 sample was determined to be statistically different from the control for survival, t-test, alpha = 0.05. The p value for the sample was 0.04, just below the significance level of 0.05. This was the only sample to show significant toxicity. Table 13 contains a summary of the survival data for these samples. Table 14 contains a summary of the routine chemical analysis data associated with these samples.

Once the tests were completed, the vials containing the fish or liver samples were stored at 4°C for 24 hours, then at -20ºC until processed for RNA isolation and gene expression analysis.

Studies 2-19, 2-20 and 2-25: The tests with the May water column and sediment samples were successful, with acceptable survival in the control samples for all tests. Survival was 100% for all animals exposed in Study 2-19, the first FHM adult gene expression test in the lake water samples, Table 25. After 24 hours of exposure, the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis. Survival was 100% for all animals exposed in Study 2-20, the first FHM adult gene expression sediment conducted with the lake sediment samples, Table 26. After 48 hours of exposure the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis. Control survival was 94.4% (Table 27) for the fry exposed to the elutriate samples prepared from the lake sediment samples (Study 2-25). Survival in the other 4 samples ranged from 88.1% to 95.0%. None were found to be statistically different from the control for survival. At the end of the test, all live fry were collected and preserved in RNAlater buffer. The tubes were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis.

Studies 2-27, 2-37 and 2-40: The tests with the June water column and sediment samples were successful, with acceptable survival in the control samples for all tests. Survival was 100% for all animals exposed in Study 2-27, the second FHM adult gene expression test in the lake water samples, Table 28. After 24 hours of exposure, the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24

hours, then moved to -20°C storage until prepared for gene expression analysis. Survival was 100% for all animals exposed in Study 2-37, the first FHM adult gene expression sediment conducted with the lake sediment samples, Table 29. After 48 hours of exposure the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis. Control survival was 98.8% (Table 30) for the fry exposed to the elutriate samples prepared from the lake sediment samples, Study 2-40. Survival in the other 4 samples ranged from 93.6% to 98.2%. None were found to be statistically different from the control for survival. At the end of the test, all live fry were collected and preserved in RNAlater buffer. The tubes were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis.

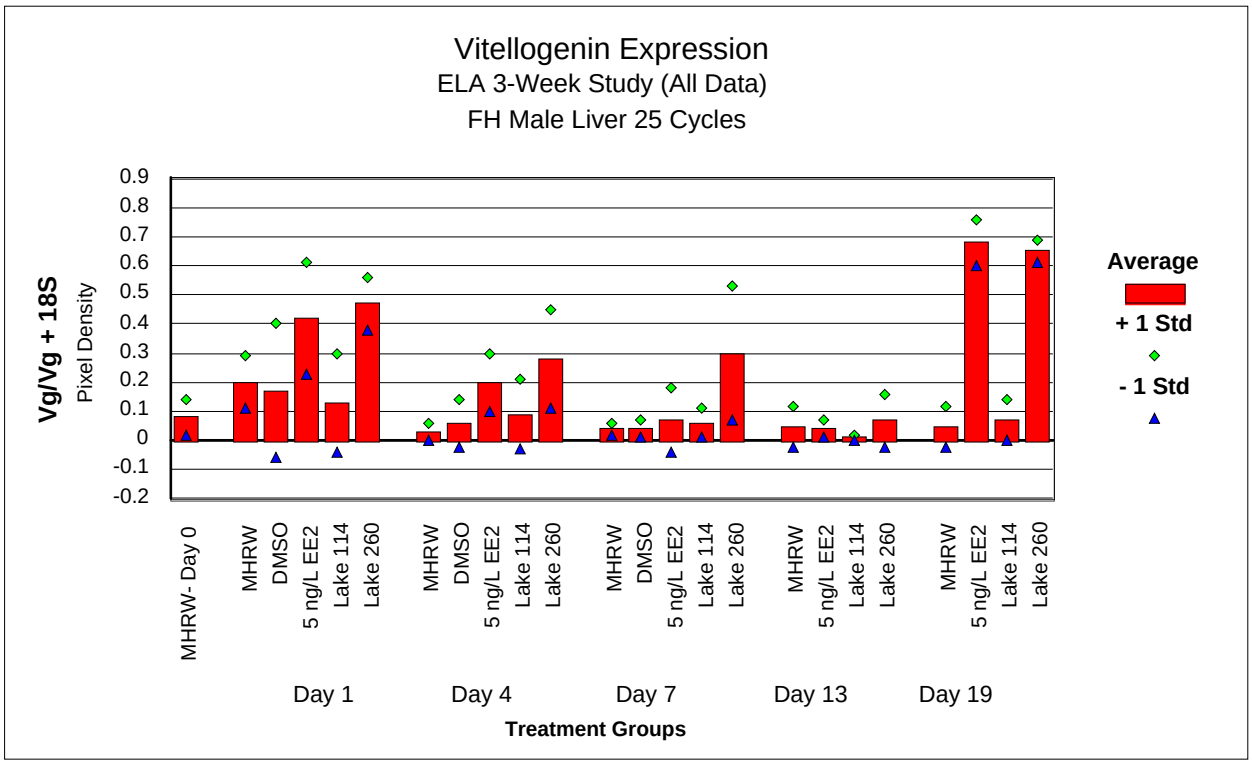
Studies 2-56, 2-64 and 2-65: The tests with the October water column and sediment samples were successful, with acceptable survival in the control samples for all tests. Survival was 100% in all but one of the samples for Study 2-56, the FHM adult gene expression test in the third set of lake water samples, Table 31. Survival was 90% in the Lake 239 water sample, due to the death of the female fish in replicate 1. After 24 hours of exposure, the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis. Control survival was 90% in Study 2-64, the FHM adult gene expression sediment conducted with this set of lake sediment samples, Table 32. This was due to the loss of the female fish in replicate 5. Survival was 100% in the remaining samples. After 48 hours of exposure the fish were sacrificed and the liver tissue collected and placed into RNAlater. The tissue samples were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression

analysis. Control survival was 97.0% (Table 33) for the fry exposed to the elutriate samples prepared from the lake sediment samples, Study 2-65. Survival in the other 4 samples ranged from 94.0% to 98.0%. None were found to be statistically different from the control for survival. At the end of the test, all live fry were collected and preserved in RNAlater buffer. The tubes were stored at 4°C for 24 hours, then moved to -20°C storage until prepared for gene expression analysis.

Study 3-37: Results from the sediment elutriate test showed excellent survival of the fry in all but Lake 260 CB (Center Buoy) treatment after 2 days of exposure. The survival in Lake 114 sediment elutriate was 99.5% and in Lake 260 SS (Shore Sediment) was 96.5%. Lake 260 CB failed however, with a survival of only 8.5%, which was found to be statistically different from that in the control, t-test, alpha = 0.05. All replicates of Lake 260 CB were composited into one tube (#21) due to the poor survival. The DMSO control survival was 99.5%, with survival in the EE2 positive control of 99.5% as well. Table 34 contains a summary of the survival data and statistical analysis results.

Study 3-43: Results from the sediment elutriate test showed acceptable survival of the fry. The survival in all exposures exceeded the 90% acceptability criteria. Tables 35 and 36 Contain routine chemistry data. Table 37 contains a summary of the survival data and statistical analysis results.

At the end of the test, the live larvae were collected and placed into labeled tubes with Tri reagent. The vials of buffer with tissue were homogenized, then the vials were then stored at -80°C until removed for RNA isolation and gene expression analysis.



The only problem encountered in all of the FHM fry tests (Appendix Tables 6-10) were with the fry exposed in the fish tanks in the mesh covered chambers. As stated previously, survival in this system was poor, with no sample meeting the minimum control survival criteria of 90% or greater. Control survival was 85%, control + DMSO was 84.4%, 5 ng/l EE2 was 65%, Lake 114 was 82.5% and Lake 260 was 85%. The fish from all treatments were collected and placed into labeled tubes of RNAlater buffer for gene expression analysis. The next test started 8/6/01, using the remainder of the samples received to start the tests. Survival was acceptable in all treatments. The next set of fry tests started when the next fresh sample (collected 8/14/01) was received on 8/15/01. At this point, the decision was made to test both the new water used for renewal and the old water from the tanks for gene expression level effects. This would provide a means to judge what effects (if any) the old water (now over 2 weeks old and in the tanks for 10 days) would have on the fry. The survival in all samples

tested, old and new 114, old and new 260, control, control+DMSO and 5 ng/l EE2, was acceptable. The next renewals, and fry tests, were performed 8/18/01, with samples collected 8/10/01 and received 8/17/01. The water left from the 8/15/01 renewals was mixed in as well. For these tests, both new and old 114 and 260 waters were tested, as well as control, control + DMSO and 5 ng/l EE2. Again, survival in all samples was acceptable. The final tests began 8/21/01. These tests included the last batch of new water for both lakes, old lake water from 8/20/01 and 8/21/01, control, control + DMSO and 5 ng/l EE2. Survival was again acceptable across all samples.

At the end of all fry tests, the fish were removed from the test vessels and transferred into labeled tubes containing RNAlater buffer. They were then stored for future RNA isolation and gene expression level analysis. Due to the lack of sufficient sample, all tests were conducted as static, non-renewal. A set of routine chemical parameters (pH, dissolved oxygen, conductivity and temperature) were determined for the new water used to start the test (initial) and this same water after the 48-hour exposure period (final).

Once the tests were completed, the vials containing the fish or liver samples were stored at 4°C for 24 hours, then at -20°C until processed for RNA isolation and gene expression analysis. Males exposed to labline water, water with DMSO, and Lake 114 water showed no vitellogenin expression (Figure 5). Males exposed to Lake 260 water showed extensive increase in vitellogenin levels, even higher in some cases than males exposed to 5 ng/L EE2. Males had variable response to both the 5ng/L water and the Lake 260 water. Two of the fish exposed to 5 ng/L EE2 showed no increase in expression of vitellogenin. One male exposed to Lake 260 water showed no expression, while two had expression levels comparable to that of fish exposed

to 5 ng/L EE2. The remaining fish had very high levels of expression, roughly five times that of the high level responders to the 5 ng/L EE2.

5 DISCUSSION

Vitellogenin gene mRNA was upregulated in indigenous male fathead minnows after only 24-hour exposure to EE2 and was still high after four months of exposure. Similarly, indigenous male pearl dace had high vitellogenin levels after four months of exposure to EE2. Female fathead minnows continued to have high vitellogenin levels into the fall, well beyond the normal breeding season, although pearl dace females in this study did not follow this trend. In females, vitellogenin protein is sequestered in eggs. In males and non-reproductive females (i.e., those without eggs) vitellogenin has no sink, and the protein continues to circulate in the blood. High levels of protein can cause problems for these fish. After four months of continuous exposure to EE2, fathead minnows exhibited widespread fibrosis and inhibited development of testicular tissues (Palace 2002). Also, EE2-exposed fish had kidney anomalies including edema within and between the kidney tubules, and hyaline deposits in the tubule cells. Liver tissues showed a loss of glycogen stores and increased liver cell size. These latter effects on the kidney and liver were likely due to the production and accumulation of VTG in these tissues.

The vitellogenin primers used for quantitative real time RT-PCR were originally designed for use with fathead minnows, the sequence of the primers having been based on the fathead minnow vitellogenin gene. These primers appeared to work well with pearl dace samples. We sequenced the pearl dace vitellogenin cDNA to determine its similarity to fathead minnow vitellogenin. The two sequences were 89% similar based on a BLAST search of the sequence. Since the sequencing was done in only one direction, more careful sequencing would likely increase the estimate of similarity.

One of the difficulties encountered in working with wild fish populations was sex determination. Determining the sex of fathead minnows from the ELA lakes was more difficult than determining sex in laboratory-reared animals. The wild fish were generally smaller than those used in lab exposures, which is likely a consequence of the very large populations that were in evidence. Male fathead minnows did not have well-developed secondary sex characteristics (swollen fat pad on the nape, nuptial tubercles around the mouth and nose) that facilitate sex determination in laboratory-reared fish. Internal anatomy (identification of gonadal tissues as ovary or testis) was of little use in diagnosing sex since the females were not in breeding condition, so eggs were not present.

Determining the sex of pearl dace was even more problematic. In this species, the only external secondary sex characteristic is a slightly thickened first spine on the pelvic fins, which is seen as a slightly darker brown anterior edge of the pelvic fin. Doubts about this method were voiced. Again, internal anatomy proved of little value in most fish, since the females were not in breeding condition, and the lack of eggs made the identification of gonads nearly impossible. For individuals with more experience, confidence in sex determination seemed higher than for us. Because of the difficulties in distinguishing between sexes, there exists the very real possibility that some of the fish that we identified as males were in fact females. The inclusion of female fish from reference lakes, with their relatively high vitellogenin levels, in the male samples, would minimize the difference in vitellogenin expression between males from dosed and undosed lakes. This would explain why some males from the reference lakes exhibited high vitellogenin levels, whereas laboratory-reared fathead males rarely show even a small level of expression when not exposed to EE2. Within a group of EE2-exposed fish it is expected that not all will respond equally. There are often high expressing fish and fish that do not respond at all.

Although trends can be seen between exposed and nonexposed fish, these differences often are not statistically significant.

6 CONCLUSIONS

Gene expression in indigenous male fathead minnows and pearl dace collected at all time intervals from the dosed lake showed a constant level of elevation. Gene expression in the 2001 fathead minnow deployment study was detected within 24 hours after deployment of control fish into the treated lake and stayed elevated for the entire 13-day study. Highly variable gene expression was found in fathead minnow fry exposed to dosed lake sediments, but no significant gene expression was found in fry exposed to reference lake sediments. Male adult fathead minnows exposed to sediment elutriates from sediments collected in 2004 in the previously dosed lake showed significant vitellogenin gene expression. Results indicate that RT-PCR analyses of total RNA can be used to provide a rapid and timely estimate of exposure to estrogenic substances.

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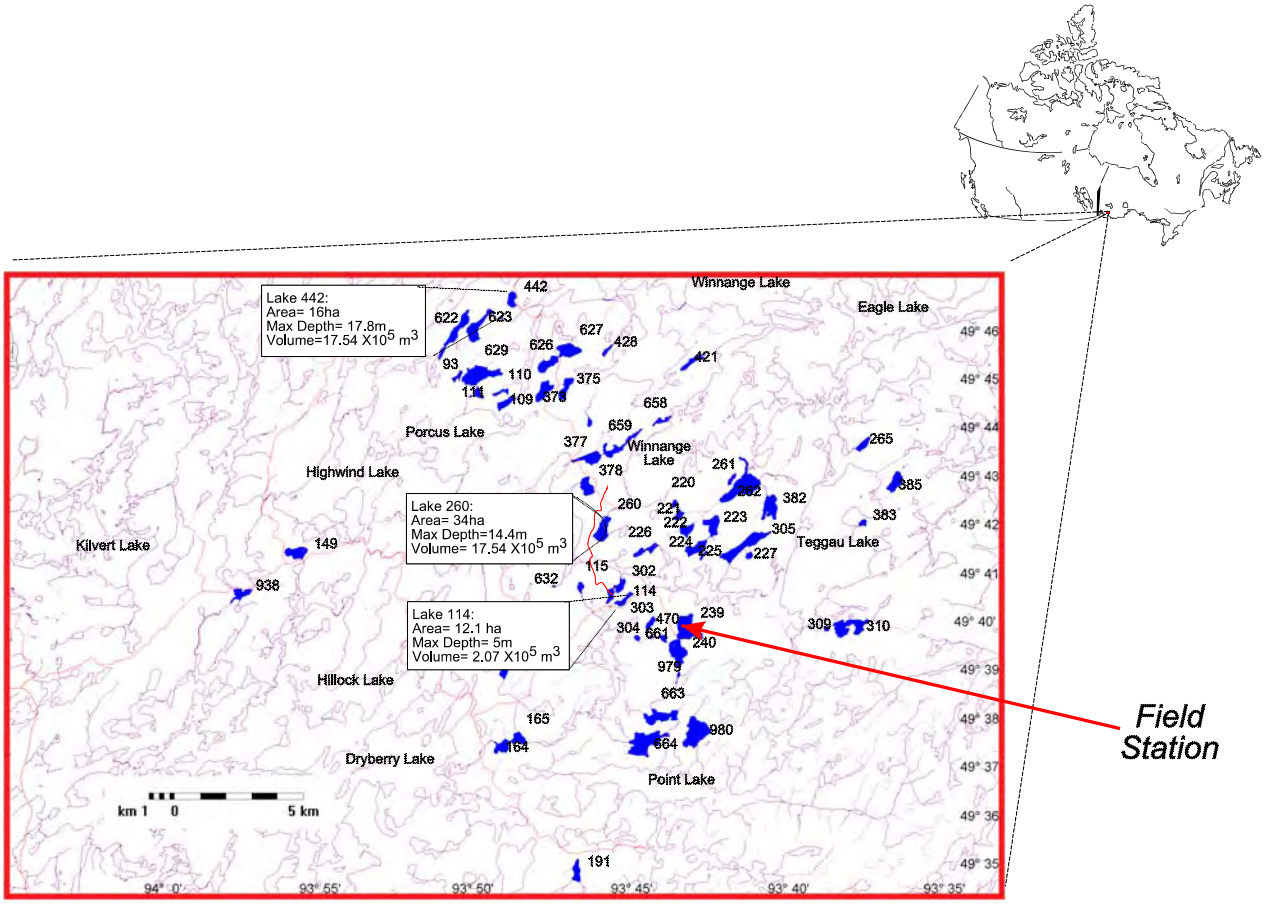

Experimental Lakes Area

Designated Research Lakes shaded Blue

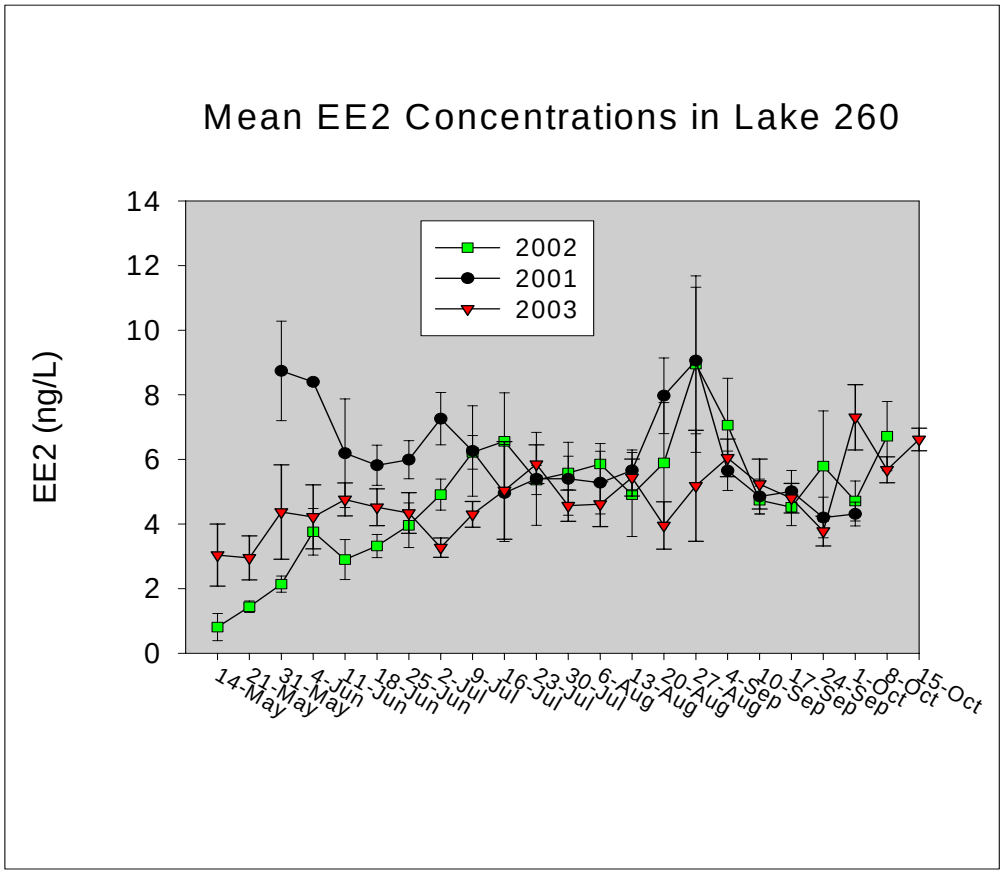

Figure 2. Concentration of EE2 (ng/L) in Lake 260 during the three years of dosing.

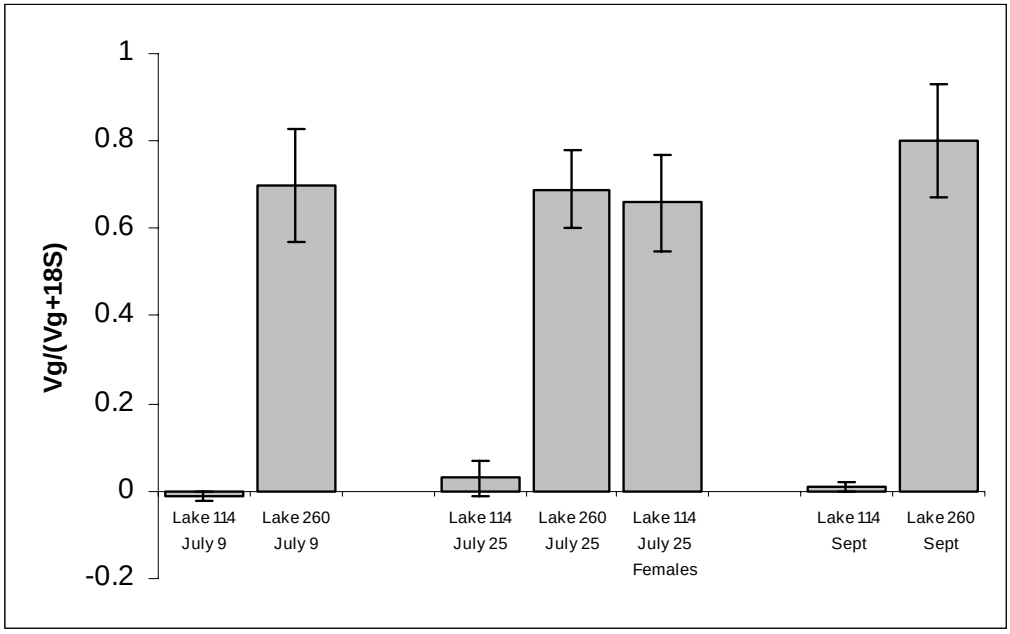

Figure 3. Study 1-21: vitellogenin gene expression in adult male fathead minnows collected from Lake 260 in 2001. PCR performed on liver for 17 cycles. Error bars represent ±1 standard deviation.

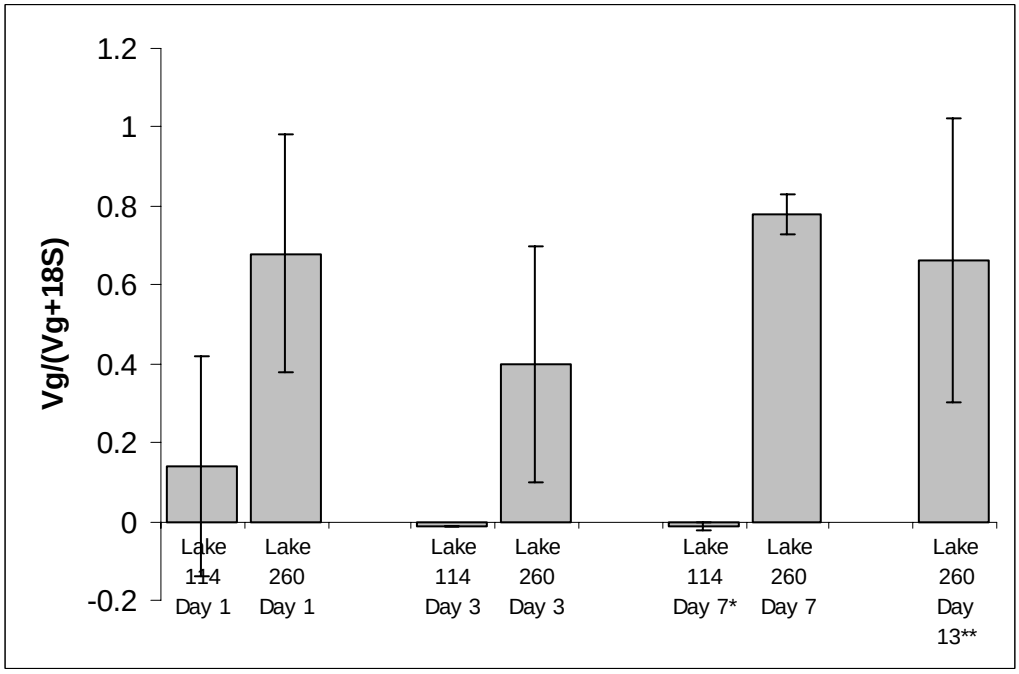

Figure 4. Study 1-21: vitellogenin gene expression in adult male fathead minnows deployed in Lake 114 and Lake 260 in 2001. PCR performed on liver for 17cycles. Each sample consisted of five fish, except *n=7; **n=4. Error bars represent ±1 standard deviation.

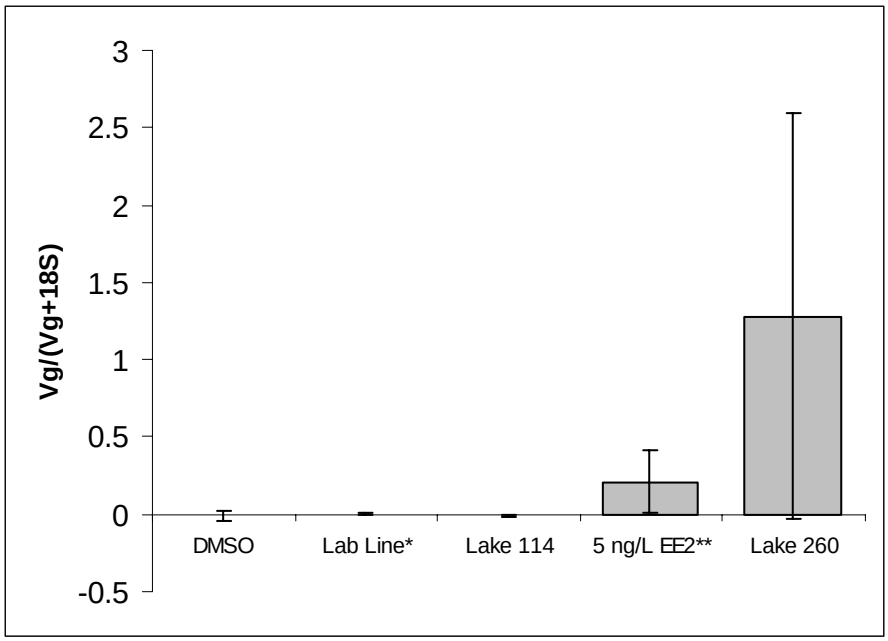

Figure 5. Study 1-11: vitellogenin gene expression in laboratory reared males exposed to water collected from ELA lakes in 2001. PCR performed on liver for 17 cycles. Each sample consisted of four fish, except *n=3; **n=5. Error bars represent ±1 standard deviation.

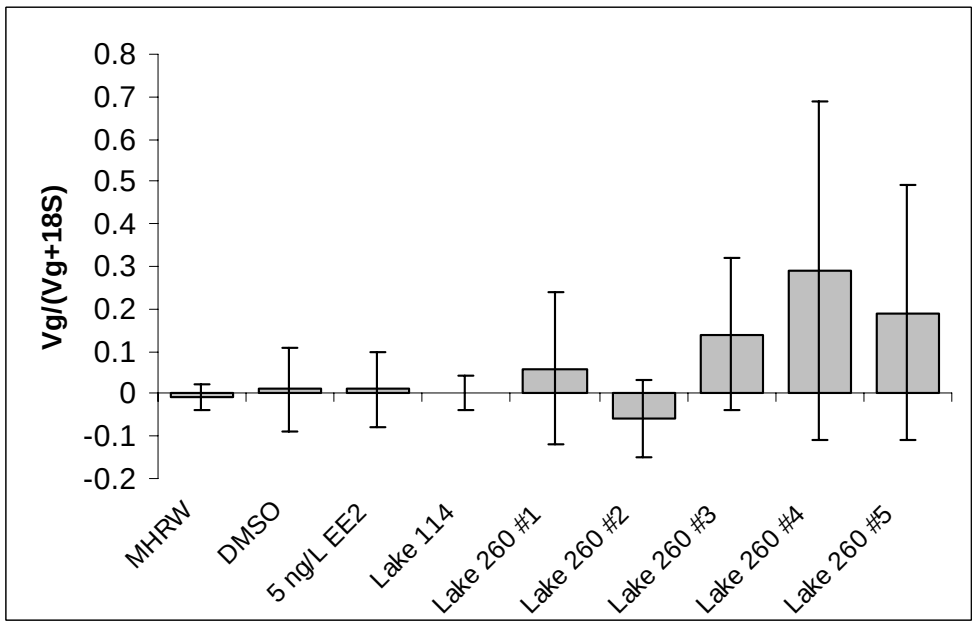

Figure 6. Study 1-26: vitellogenin gene expression in fry exposed to elutriate prepared from water collected from Lake 114 and Lake 260 in June of 2002. PCR performed on whole fry homogenate for 32 cycles. Each sample consisted of four replicates of a pool of forty fry. Error bars represent ±1 standard deviation.

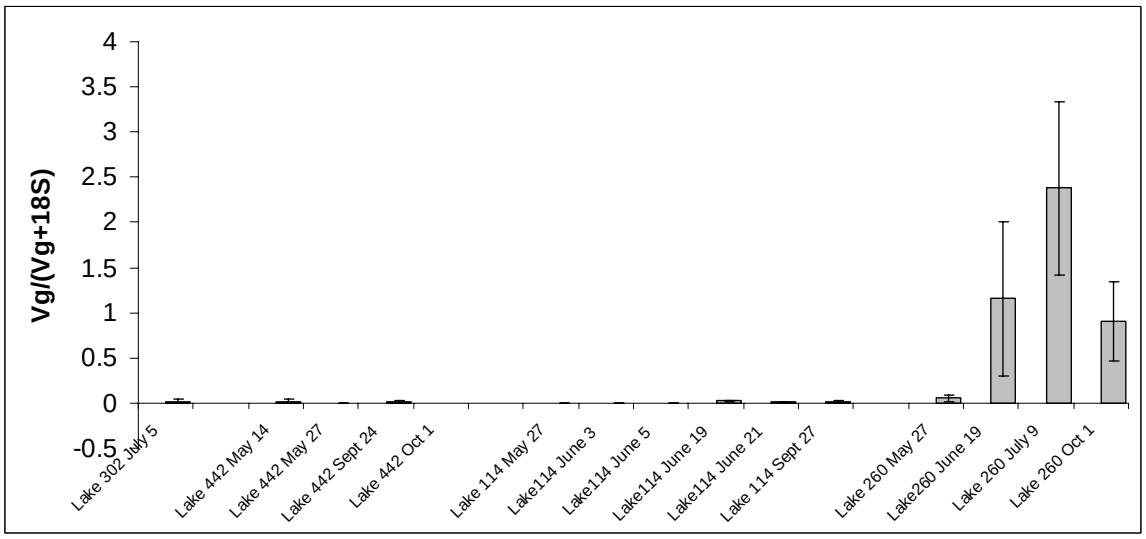

Figure 7. Study 2-62: vitellogenin gene expression in male fathead minnows collected in 2002. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

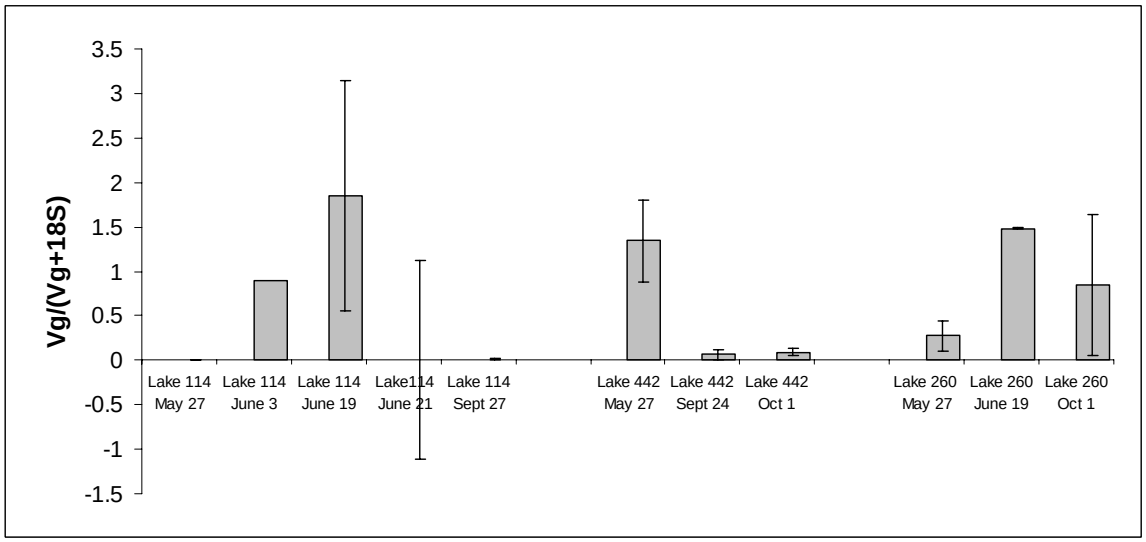

Figure 8. Study 2-62: vitellogenin gene expression in female fathead minnows collected in 2002. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

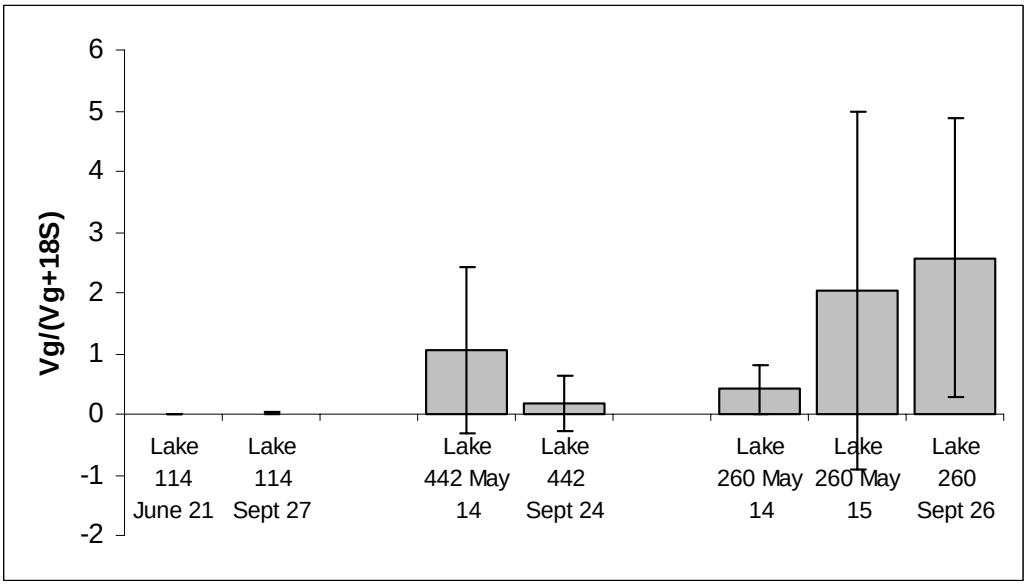

Figure 9. Study 2-63: vitellogenin gene expression in male pearl dace collected in 2002. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

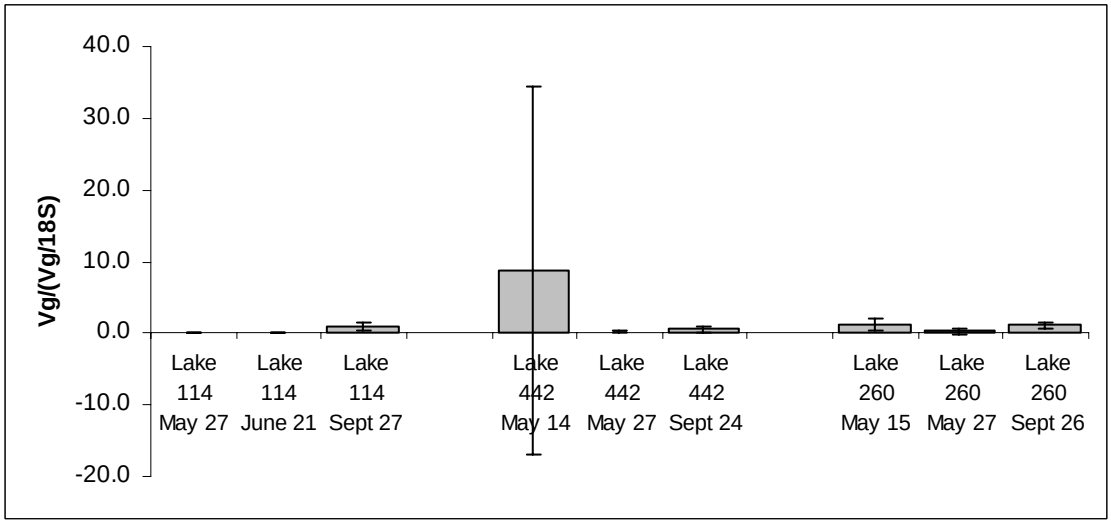

Figure 10. Study 2-63: vitellogenin gene expression in female pearl dace collected in 2002. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

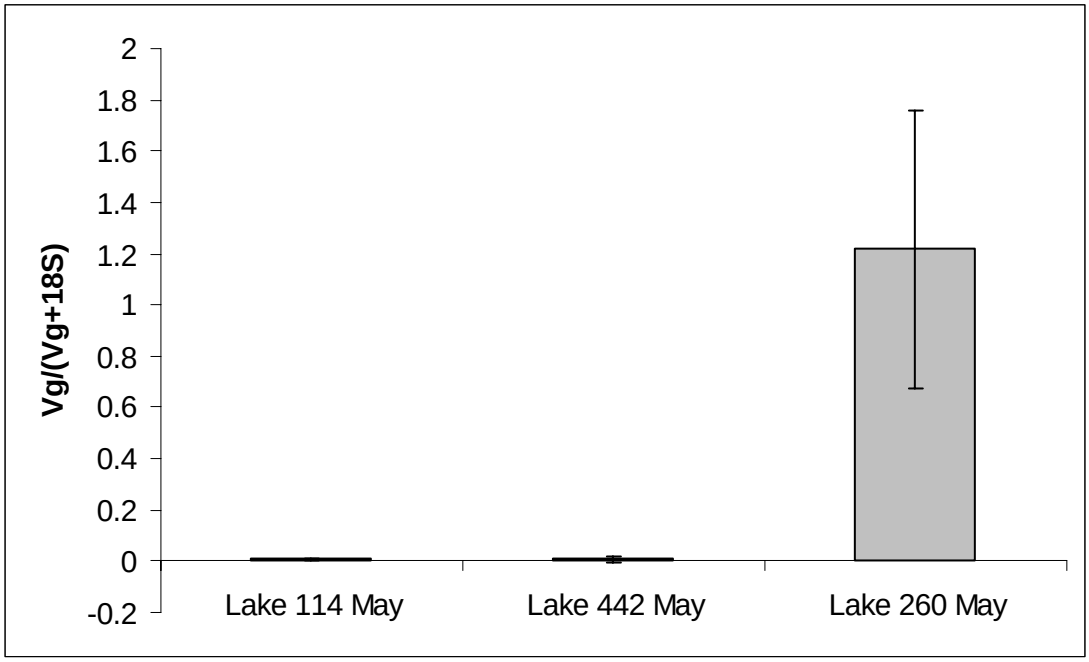

Figure 11. Study 3-45: vitellogenin gene expression in male fathead minnows collected in May 2003. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

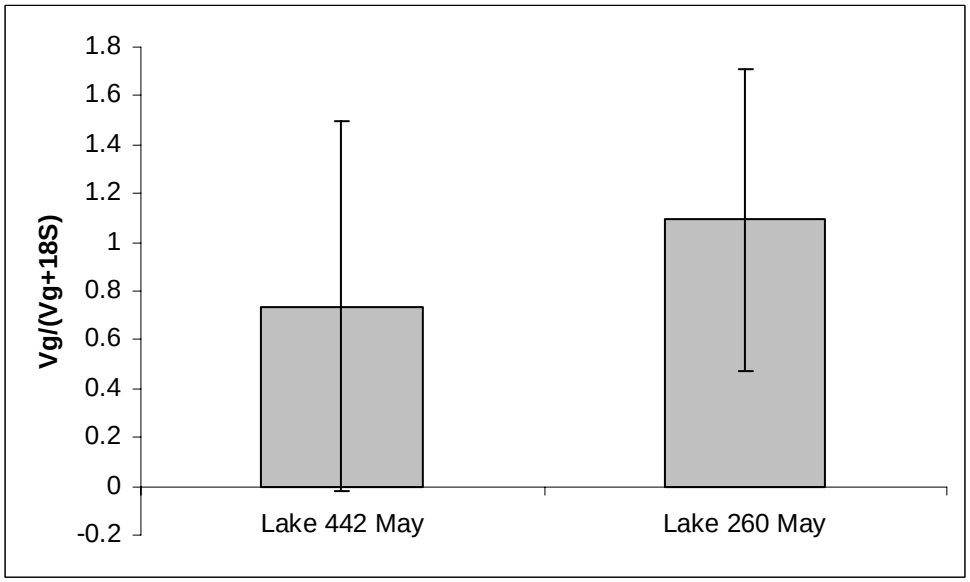

Figure 12. Study 3-45: vitellogenin gene expression in female fathead minnows collected in May 2003. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

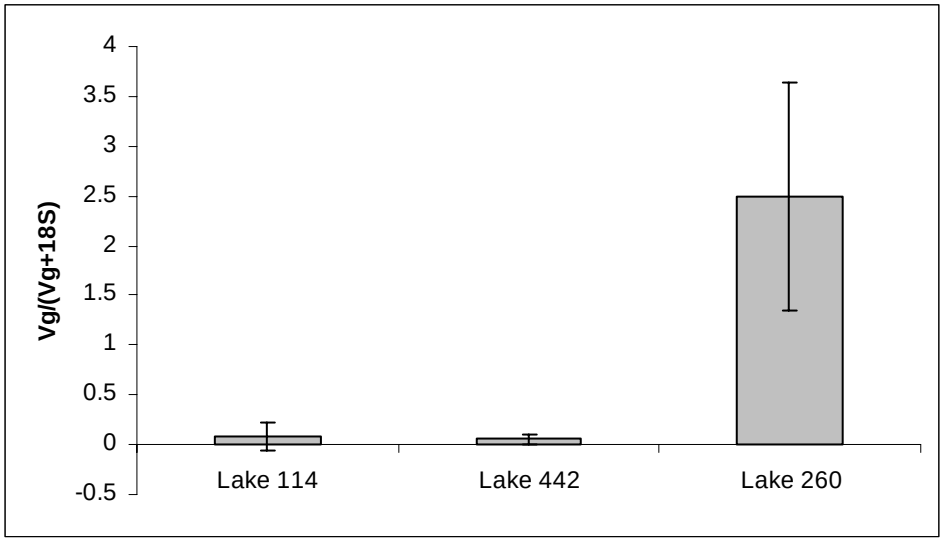

Figure 13. Study 3-45: vitellogenin gene expression in male pearl dace collected in May 2003. PCR performed on liver for 17cycles. Lake 114: n=8; Lake 442 and 260: n=6. Error bars represent ±1 standard deviation.

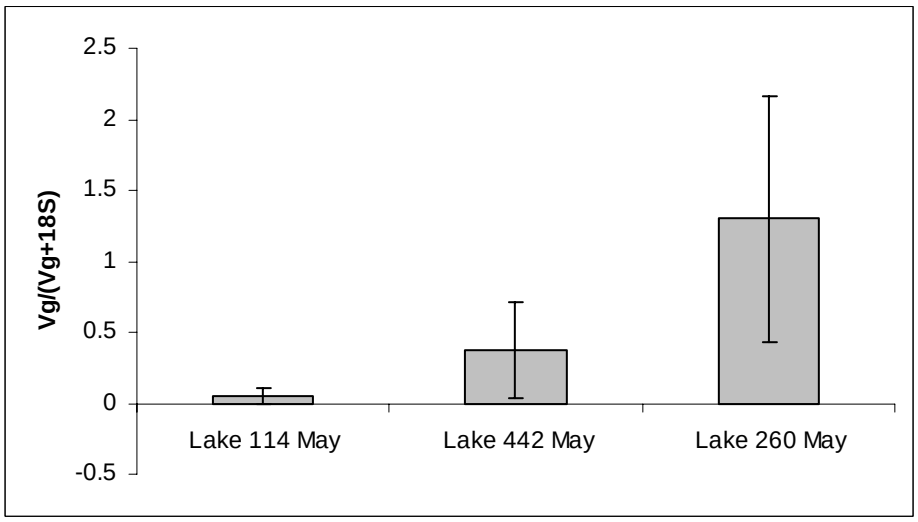

Figure 14. Study 3-45: vitellogenin gene expression in female pearl dace collected in May 2003. PCR performed on liver for 17cycles. Error bars represent ±1 standard deviation.

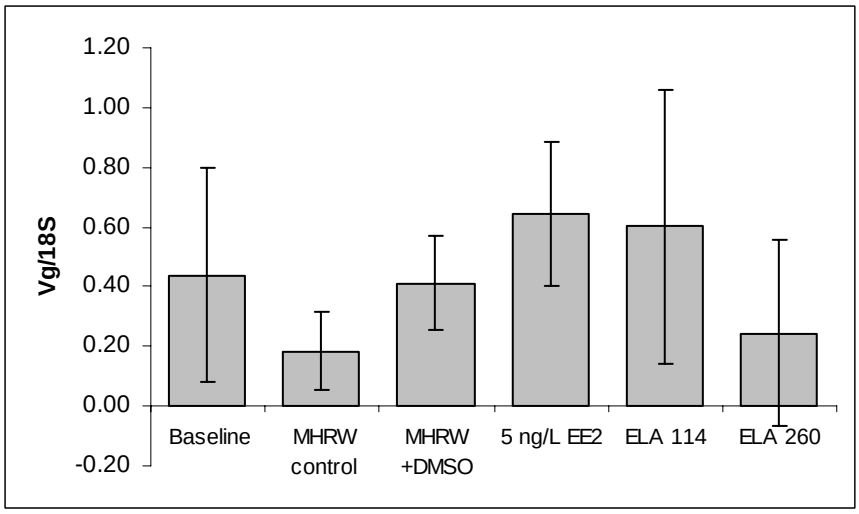

Figure 15. Study 4-19: quantitative real time PCR analysis of vitellogenin gene expression in female fathead minnows collected in May 2004. Sample size is five for each group. Error bars represent ±1 standard deviation.

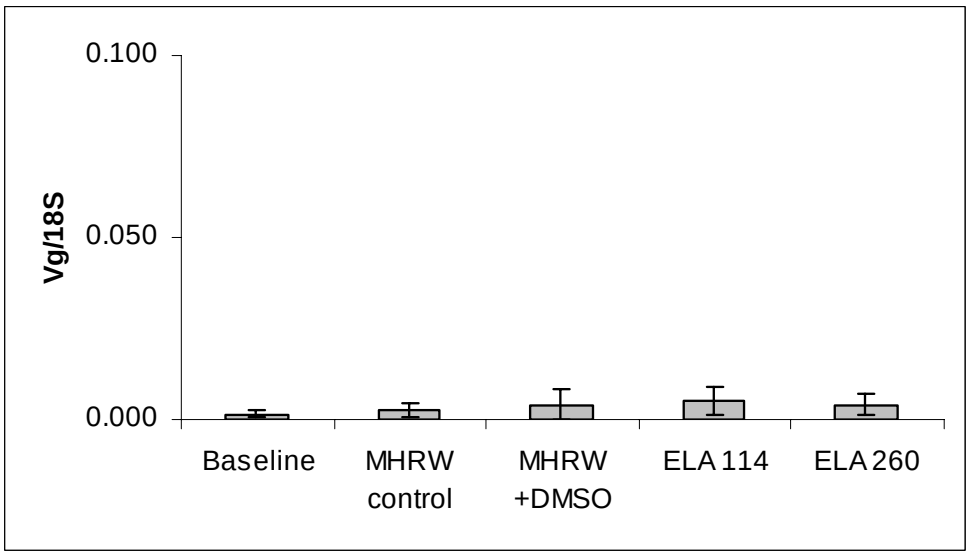

Figure 16. Study 4-19: quantitative real time PCR analysis of vitellogenin gene expression in male fathead minnows collected in 2004. Sample size is five for each group. Error bars represent ±1 standard deviation.

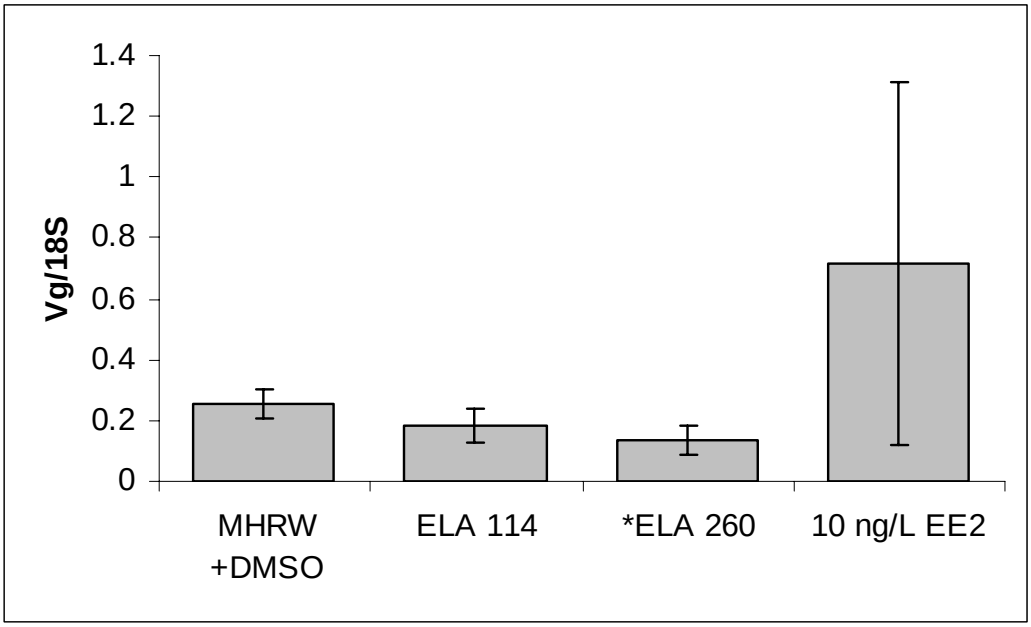

Figure 17. Study 4-21: quantitative real time PCR analysis of vitellogenin gene expression in fathead minnow fry exposed to elutriate prepared from sediment collected in 2004. Sample size is five except *n=4. Error bars represent ±1 standard deviation.

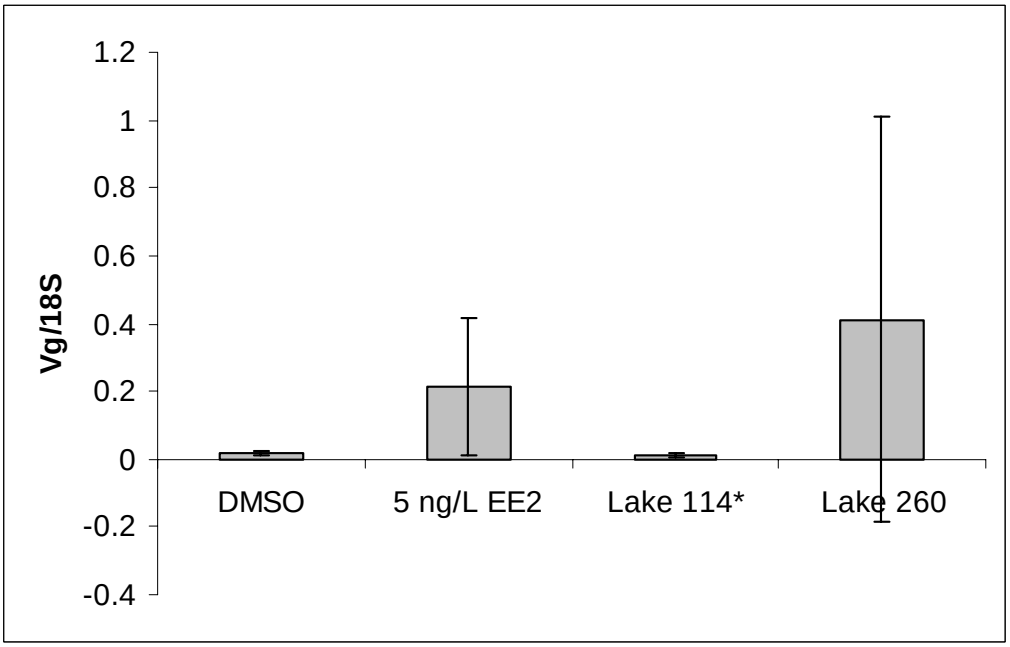

Figure 18. Study 4-20: quantitative real time PCR analysis of vitellogenin gene expression in male fathead minnows exposed to elutriate prepared from sediment collected in 2004. Sample size is six except *n=5. Error bars represent ±1 standard deviation.

Appendix 1

The description of the EE2 dosing of the ELA lake from the Department of Fisheries and Oceans Canada project plan, courtesy of Karen Kidd:

Despite the overt physiological evidence that fish are being adversely impacted by endocrine disrupting substance (EDS), it remains unclear whether these compounds are affecting aquatic organisms at the population level. It has been recognized nationally and internationally that there is a need to determine whether the molecular and cellular effects, such as egg protein (vitellogenin) production in males, observed in fish exposed to estrogen mimics are indicative of changes in the viability of the population. Though significant progress has been made in characterizing the effects of estrogenic contaminants in individuals, population level approaches to identify and quantify effects are lacking (US EPA 1997, Jobling et al. 1998). The following study has been designed to address this need, and to provide information that will complement lab and field studies being conducted by DFO, other government agencies, and academic institutions.

We are conducting a multi-year whole-ecosystem study at the Experimental Lakes Area (ELA) in northwestern Ontario that will expose well-defined aquatic populations to the synthetic estrogen 17-alpha-ethynylestradiol (EE2). This study has been designed to determine whether aquatic populations are adversely impacted by an EDS, and to validate and calibrate the relationship between organism- and population-level responses to hormone mimics. EE2 was chosen for this experiment because it is known to be a potent estrogen mimic in fish and other vertebrates (Routledge et al. 1998), and it is found at low ng/L levels in river waters downstream of sewage treatment plants (Belfroid et al. 1999).

In the first two years of this study, baseline information was collected on the populations of fish, amphibians, benthic and pelagic invertebrates, microorganisms and algae in the study (Lake 260) and reference lakes (Lakes 375, 114, 305, 224 and 442). For three consecutive summers (2001 through 2003), EE2 was added continuously to Lake 260 over the ice-free season to maintain the water at a concentration known to induce biochemical responses in fish (5 to 6 ng EE2 L-1). This lake was chosen for the EE2 amendments because it contains lake trout, white sucker, pearl dace and fathead minnows (a commonly used species in EDS assays), allowing for contrasts between large and small fish species. It also has long-term records on fish populations (since 1984), and historical water quality and zooplankton data. Impacts of EDS exposure on small and large fish populations will be assessed using age and size distributions, sex ratios, age to maturity, condition factors, abundance, growth rates, annual survival and recruitment, and fertilization studies. Organism-level responses in fish are also being examined and include impacts on circulating and tissue hormone levels, gonadal development (histological assessments) and in vitro steroid synthesis, and vitellogenin production. These responses will be used to evaluate the utility of the different exposure markers commonly used in field screening programs, and will contribute to our understanding of the underlying mechanisms that mediate a fish's response to estrogenic compounds. Changes in the populations of algae, microorganisms, zooplankton, benthic invertebrates and tadpoles will also be evaluated after EE2 amendments, using both baseline and reference lake data collected this past field season and over the next

several years. In summary, this study will provide much-needed information on the magnitude of the effects of a potent EDS on aquatic populations.

Appendix 2

Table 10. Results from a FHM fry gene induction test performed on 6-22-01 with moderately hard reconstituted water (MHRW), labline water, 5 ng/l EE2, Lake 114 and Lake 260. Test duration of 48 hours. Lake 114 and 260 samples collected 6-20-01. **Study # 1-12**.

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**exp**	**live**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	160	156	8.00	8.16	8.3	7.9	300	331	24.2	24.1
Labline	1-4	160	155	8.07	7.92	8.0	7.9	452	395	24.1	24.2
5 ng/l EE2	1-4	160	156	8.01	8.12	8.3	8.0	298	422	24.0	24.0
Lake 114	1-4	160	133	6.67	7.01	8.4	8.0	14	36	24.0	24.3
Lake 260	1-4	160	143	6.75	7.07	8.8	8.1	21	46	24.2	24.0

Table 11. Results from a FHM adult male gene induction test performed on 6-22-01 with moderately hard reconstituted water (MHRW), labline water, 5 ng/l EE2, Lake 114 and Lake 260. These tests used 1 fish in each of the 5 test replicates. Test duration of 24 hours. Lake 114 and Lake 260 samples collected 6-20-01. **Study # 1-11**.

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**exp**	**live**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-5	5	5	8.00	7.73	8.3	6.9	300	308	24.2	24.2
Labline	1-5	5	5	8.07	7.77	8.0	6.9	452	464	24.1	24.1
5 ng/l EE2	1-5	5	5	8.01	7.72	8.3	6.8	298	306	24.0	24.1
Lake 114	1-5	5	5	6.67	6.98	8.4	6.8	14	26	24.0	24.1
Lake 260	1-5	5	5	6.75	8.86	8.8	6.6	21	32	24.2	24.1

Table 12. Results from sediment elutriate tests conducted with Canadian ELA samples using *P. promelas* embryo/larvae. Elutriate samples were prepared after the embryo-larval tests on the whole sediment resulted in 100% mortality for the lake sediments. Tests were 5 days duration, conducted at 25°C. Water renewed daily. Tests included MHRW control, MHRW + DMSO solvent blank, 5 ng/l EE2 positive control and six lake samples. **Study #1-26.**

Conc./Site I.D.	rep	exp	live	RNAlater Tube #	% Sur	CV	t-test*
Lab Cont	1-4	160	156	1-4	97.5	2.09	N/A
MHRW + DMSO	1-4	160	151	5-8	94.4	2.54	0.094
5 ng/l EE2	1-4	160	148	9-12	92.5	2.21	0.073
Lake 114	1-4	160	127	13-16	79.4	25.51	0.125
Lake 260 #1	1-4	160	113	17-20	70.6	29.24	**0.041**
Lake 260 #2	1-4	160	151	21-24	94.4	4.52	0.235
Lake 260 #3	1-4	160	134	25-28	83.8	19.88	0.152
Lake 260 #4	1-4	160	139	29-32	86.9	11.60	0.084
Lake 260 #5	1-4	160	121	33-36	75.6	29.37	0.097

* Samples analyzed using t-test function in Lotus, alpha = 0.05. Site I.D. and t-test result in bold denotes sample found to be statistically different from the control.

Table 13. Routine initial and final chemical analysis of elutriate samples prepared from Canadian ELA Lake samples. Listed are the initial chemical determination for the start of the test and the ranges of final chemistry results for each analyte. **Study # 1-26**.

	pH		DO		Conductivity		Temp	
Conc.	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	8.32	8.14- 8.46	8.2	7.4- 8.1	319	317- 345	25.1	24.8- 25.6
MHRW + DMSO	8.28	8.11- 8.26	8.3	7.1- 7.8	314	318- 333	25.2	24.9- 25.5
5 ng/l EE2	8.31	8.10- 8.23	8.2	7.3- 7.6	312	319- 336	25.2	25.0- 25.5
Lake 114	7.37	7.53- 7.94	7.8	7.2- 7.8	235	249- 262	24.7	24.9- 25.4
Lake 260 #1	6.97	7.47- 7.74	8.5	7.1- 7.7	214	224- 242	24.4	24.8- 25.3
Lake 260 #2	6.96	7.33- 7.68	8.4	7.1- 7.8	234	235- 268	25.0	24.9- 25.2
Lake 260 #3	6.58	7.30- 7.53	8.5	7.0- 7.8	230	230- 259	24.9	25.0- 25.3
Lake 260 #4	6.64	7.33- 7.48	8.5	7.2- 7.8	202	212- 251	24.9	24.8- 25.2
Lake 260 #5	6.65	7.29- 7.50	8.5	7.4- 7.8	215	228- 238	24.8	24.9- 25.1

Table 14. Results from the Day 1 necropsy of fish for the Canadian ELA 3-week study (8/2/01). Fish were randomly selected for use in the necrospy procedure and water samples were collected to analyze for final water chemistries. **Study #1-23.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**# N**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1	5	6-10	8.01	7.91	8.1	7.4	301	323	24.2	24.6
Cont + DMSO	1	5	16-20	8.04	7.68	8.0	6.8	303	313	24.4	24.8
5 ng/l EE2	1	5	11-15	8.09	7.74	8.1	7.0	304	317	24.3	25.0
Lake 114	1	5	21-25	6.65	7.55	8.9	6.9	50	65	24.2	24.5
Lake 260	1	5	26-30	7.13	7.18	8.4	6.3	62	52	24.2	24.6

N = number necropsied.

Table 15. Results from the Day 4 necropsy of fish for the Canadian ELA 3-week study (8/6/01). Fish were randomly selected for use in the necrospy procedure and water samples were collected to analyze for final water chemistries. Water was changed at this time. **Study #1-23**.

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**# N**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1	5	31-35		7.66		7.7		372		24.8
Cont + DMSO	1	5	36-40		7.57		7.7		372		24.7
5 ng/l EE2	1	5	41-45		7.69		7.8		364		24.9
Lake 114	1	5	46-50		7.14		7.8		104		24.6
Lake 260	1	5	51-55		7.17		7.8		113		24.7

Table 16. Results from the Day 7 necropsy of fish for the Canadian ELA 3-week study (8/9/01). Fish were randomly selected for use in the necrospy procedure and water samples were collected to analyze for final water chemistries. Water was not changed at this time. **Study #1-23.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**# N**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1	5	56-60		7.99		7.7		345		24.6
Cont + DMSO	1	5	61-65		7.96		7.7		371		24.7
5 ng/l EE2	1	5	66-70		8.09		7.8		372		24.6
Lake 114	1	5	71-75		7.58		7.8		78		24.3
Lake 260	1	5	76-80		7.61		7.8		82		24.4

Table 17. Results from the Day 13 necropsy of fish for the Canadian ELA 3-week study (8/15/01). Fish were randomly selected for use in the necrospy procedure and water samples were collected to analyze for final water chemistries. Water was changed at this time. No data for DMSO control, all fish dead by this point. **Study #1-23.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**# N**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1	5	81-85		8.28		7.9		381		24.0
Cont + DMSO	1	5	N/A		N/A		N/A		N/A		N/A
5 ng/l EE2	1	5	86-90		8.15		7.8		402		24.2
Lake 114	1	5	91-95		7.93		7.8		102		24.3
Lake 260	1	5	96-100		7.70		7.6		125		24.3

Table 18. Results from the Day 20 necropsy of fish for the Canadian ELA 3-week study (8/21/01). Fish were randomly selected for use in the necrospy procedure and water samples were collected to analyze for final water chemistries. This completed the study. All remaining fish were necropsied at this time. **Study #1-23.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**# N**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1	10	101-110		7.88		7.1		354		24.5
Cont + DMSO	1	0	N/A		N/A		N/A		N/A		N/A
5 ng/l EE2	1	8	111-118		7.68		7.2		363		24.4
Lake 114	1	10	119-128		7.78		6.8		100		24.8
Lake 260	1	5	129-133		7.63		7.0		121		24.6

Table 19. Results from the first fry test with fresh samples from Lakes 114 and 260, 8/2/01. Fry were exposed in the fish tanks in screen covered PVC exposure chambers. Tests were 48 hours duration. No renewal of test solution. Fry used were 24 to 48 hours old. As is apparent, survival was below acceptable levels, so future tests were conducted in 500 ml glass beakers. **Study #1-24.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**sur**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	136/160	1-4	8.10	7.75	8.0	7.6	315	338	24.1	24.8
Cont + DMSO	1-4	135/160	5-8	8.08	7.72	8.0	7.5	310	335	24.2	24.9
5 ng/l EE2	1-4	104/160	9-12	8.00	7.64	8.0	7.2	311	329	24.1	25.1
Lake 114	1-4	132/160	13-16	7.78	7.14	8.4	6.7	61	71	24.1	24.6
Lake 260	1-4	136/160	17-20	7.24	7.16	8.5	6.7	50	81	24.2	24.8

Table 20. Results from the second fry test with samples from Lakes 114 and 260, 8/6/01. Fry were exposed in glass beakers, 300 ml of test solution. Tests were 48 hours duration. No renewal of test solution. Fish used were fry 24 to 48 hours old. **Study #1-24.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**sur**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	159/160	21-24	8.32	8.33	8.4	7.8	280	306	24.8	25.1
Cont + DMSO	1-4	160/160	25-28	8.13	8.25	8.4	7.5	287	306	24.8	25.2
5 ng/l EE2	1-4	160/160	29-32	8.13	8.17	8.2	7.5	287	312	24.7	24.9
Lake 114	1-4	158/160	33-36	6.92	7.83	8.2	7.4	17	60	24.9	24.6
Lake 260	1-4	159/160	37-40	7.05	7.70	8.4	7.5	38	83	24.9	24.4

Table 21. Results from the third fry test with samples from Lakes 114 and 260, 8/15/01. For this test, both the water used to renew the tanks and the old water from the tanks was analyzed using the fry exposure test method. Fry were exposed in glass beakers, 300 ml of test solution. Tests were 48 hours duration. No renewal of test solution. Fish used were fry 24 to 48 hours old. **Study #1-24.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**sur**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	160/160	41-44	8.22	8.80	8.5	7.5	290	314	24.3	25.2
Cont + DMSO	1-4	159/160	45-48	8.22	8.71	8.2	7.5	293	310	24.4	25.2
5 ng/l EE2	1-4	160/160	53-56	8.22	8.25	8.5	7.1	290	310	24.1	24.6
Lake 114 new	1-4	160/160	57-60	7.58	7.26	8.2	7.2	21	32	24.3	24.6
Lake 260 new	1-4	160/160	61-64	7.50	7.48	8.4	7.4	14	46	24.1	24.7
Lake 114 old	1-4	160/160	49-52	7.93	8.28	7.8	7.9	102	121	24.3	25.0
Lake 260 old	1-4	148/160	65-68	7.70	7.24	7.9	6.2	125	134	24.4	24.5

Table 22. Results from the fourth fry test with samples from Lakes 114 and 260, 8/18/01. For this test, both the water used to renew the tanks and the old water from the tanks were analyzed using the fry exposure test method. Fry were exposed in glass beakers, 300 ml of test solution. Tests were 48 hours duration. No renewal of test solution. Fish used were fry 24 to 48 hours old. **Study #1-24.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**sur**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	160/160	69-72	8.01	8.26	8.0	7.6	300	3307	24.1	25.1
Cont + DMSO	1-4	160/160	73-76	8.05	8.16	8.1	7.5	304	300	24.0	25.0
5 ng/l EE2	1-4	159/160	85-88	8.10	7.94	8.0	7.2	301	301	24.2	25.0
Lake 114 new	1-4	159/160	81-84	7.43	7.71	8.8	7.3	20	48	24.1	25.0
Lake 260 new	1-4	159/160	89-92	7.40	7.00	8.7	7.0	15	80	24.3	24.9
Lake 114 old	1-4	160/160	77-80	7.78	7.72	6.8	7.0	100	85	24.2	25.0
Lake 260 old	1-4	160/160	93-96	7.63	7.31	7.0	7.0	121	45	24.4	25.0

Table 23. Results from the fourth fry test with samples from Lakes 114 and 260, 8/21/01. For this test, both the water used to renew the tanks and the old water from the tanks were analyzed using the fry exposure test method. Fry were exposed in glass beakers, 300 ml of test solution. Tests were 48 hours duration. No renewal of test solution. Fish used were fry 24 to 48 hours old. **Study #1-24.**

				pH		DO		Conductivity		Temp	
Conc.	**rep**	**sur**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
Lab Cont	1-4	159/160	97-100	8.43	8.14	8.1	7.6	293	310	24.2	24.7
Cont + DMSO	1-4	160/160	101-104	8.42	8.20	8.1	7.7	298	326	24.8	25.2
5 ng/l EE2	1-4	156/160	105-108	8.35	8.17	8.2	7.5	300	317	24.6	25.2
Lake 114 new	1-4	148/160	113-116	7.06	7.50	8.3	7.7	19	46	24.4	24.7
Lake 260 new	1-4	159/160	109-112	7.54	7.56	8.4	7.7	26	47	24.3	24.6
Lake 114 8/20 old	1-4	159/160	117-120	7.24	7.67	7.9	7.7	30	83	24.4	25.2
Lake 260 8/20 old	1-4	159/160	121-124	7.13	7.41	7.8	7.1	44	88	24.3	25.1
Lake 114 8/21 old	1-4	160/160	125-128	7.26	7.47	8.0	7.4	21	65	24.8	24.6
Lake 260 8/21 old	1-4	153/160	129-132	7.16	7.21	8.0	7.0	33	68	24.6	24.7

Table 24. Results for the Adult FHM Gene Expression test with 2 ELA Lake samples. Conducted 5/23/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and samples from Lake 114 and 260. Duration was 24 hours. **Study #2-19.**

						pH		DO		Conductivity		Temp	
Conc	**Rep**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	1-5	8.03	7.57	8.3	5.5	300	307	25.0	24.1
MHRW	1-5	f	5	5	21-25								
Cont + DMSO	1-5	m	5	5	6-10	8.08	7.96	8.3	7.7	297	314	25.0	24.0
Cont + DMSO	1-5	f	5	5	26-30								
5 ng/l EE2	1-5	m	5	5	11-15	8.10	7.75	8.5	7.2	290	304	25.0	24.2
5 ng/l EE2	1-5	f	5	5	31-35								
Lake 114	1-5	m	5	5	16-20	7.53	7.78	10.2	7.0	18	32	24.2	24.4
Lake 114	1-5	f	5	5	46-50								
Lake 260	1-5	m	5	5	21-25	7.37	7.58	10.4	6.9	22	43	24.0	24.5
Lake 260	1-5	f	5	5	51-55								
Day 0 Initial Fish	1-5	m	5	5	46-50	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	41-45	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 25. Results for the Adult FHM Gene Expression test with 2 ELA Lake sediment samples. Conducted 5/29/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and sediment samples from Lake 114 and 260. Duration was 48H. **Study #2-20.**

						pH		DO		Conductivity		Temp	
Conc	**Rep#**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	11-15	8.28	8.00	8.2	7.4	308	329	24.1	24.6
MHRW	1-5	f	5	5	36-40								
Cont + DMSO	1-5	m	5	5	16-20	8.16	8.01	8.2	7.6	306	328	24.5	24.4
Cont + DMSO	1-5	f	5	5	41-45								
5 ng/l EE2	1-5	m	5	5	21-25	8.12	7.85	8.0	6.5	306	328	24.5	24.3
5 ng/l EE2	1-5	f	5	5	46-50								
Lake 114 sediment	1-5	m	5	5	26-30	6.93	7.28	5.8	4.0	242	241	24.6	24.5
Lake 114 sediment	1-5	f	5	5	51-55								
Lake 260 sediment	1-5	m	5	5	31-35	6.68	7.11	2.9	3.9	225	211	24.5	24.4
Lake 260 sediment	1-5	f	5	5	56-60								
Day 0 Initial Fish	1-5	m	5	5	1-5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	6-10	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

** Sample 1, Lake 260, no eggs.

Table 26. Results from the *P. promelas* (FHM) embryo/larval test using elutriate samples prepared from the Canadian ELA Lakes 114 and 260 Sediments. Conducted 6/13/02. Test used 50 ml of test solution, 40 FHM eggs/replicate, MHRW control, 5 ng/l EE2 positive control and 2 ELA samples. Duration was 5 days. **Study #2-25.**

						pH		**DO**		**Conductivity**		**Temp**	
Conc.	**Rep #**	**% Sur**	**CV**	**p value**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-4	94.4	1.3	n/a	1-4	8.28	8.17	8.6	8.4	316	368	24.0	24.3
Cont + DMSO	1-4	95.0	0	.686	5-8	8.16	7.97	8.2	8.2	313	324	24.0	24.6
5 ng/l EE2	1-4	93.1	4.6	.595	9-12	8.16	7.93	8.3	8.2	315	330	24.1	24.7
ELA Lake 114	1-4	88.1	6.3	.070	13-16	7.05	7.68	9.3	8.2	228	241	25.0	24.8
ELA Lake 260	1-4	93.8	6.3	1.00	14-20	6.19	7.33	9.6	8.2	202	212	24.9	24.9

Table 27. Results for the Adult FHM Gene Expression test with Canadian ELA samples. Conducted 6/20/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and a two Canadian ELA samples. Duration was 48H. **Study #2-27.**

						pH		DO		Conductivity		Temp	
Conc	**Rep#**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	36-40	7.77	7.86	8.7	8.4	322	328	24.3	24.6
MHRW	1-5	f	5	5	11-15								
Cont + DMSO	1-5	m	5	5	41-45	7.86	7.79	8.8	8.3	322	327	24.1	24.8
Cont + DMSO	1-5	f	5	5	16-20								
5 ng/l EE2	1-5	m	5	5	46-50	7.87	7.81	8.8	6.2	319	329	24.0	25.1
5 ng/l EE2	1-5	f	5	5	21-25								
Lake 114	1-5	m	5	5	51-55	5.88	7.15	9.1	8.0	13	34	24.7	25.0
Lake 114	1-5	f	5	5	26-30								
Lake 260	1-5	m	5	5	56-60	6.57	7.17	9.1	7.9	21	37	24.6	25.1
Lake 260	1-5	f	5	5	31-35								
Day 0 Initial Fish	1-5	m	5	5	6-10	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	1-5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 28. Results for the Adult FHM Gene Expression test with 2 ELA Lake sediment samples conducted 7/24/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and sediment samples from Lake 114 and 260. Duration was 48 hours. **Study #2-37.**

						pH		DO		Conductivity		Temp	
Conc	**Rep#**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	36-40	8.14	8.01	8.3	8.0	349	339	24.8	24.5
MHRW	1-5	f	5	5	11-15								
Cont + DMSO	1-5	m	5	5	41-45	8.08	8.00	8.4	8.0	318	327	24.3	24.7
Cont + DMSO	1-5	f	5	5	16-20								
5 ng/l EE2	1-5	m	5	5	46-50	8.09	7.96	8.5	8.0	318	324	24.5	24.9
5 ng/l EE2	1-5	f	5	5	21-25								
Lake 114 sediment	1-5	m	5	5	51-55	7.08	7.62	7.8	7.8	238	223	24.4	24.8
Lake 114 sediment	1-5	f	5	5	31-35								
Lake 260 sediment	1-5	m	5	5	56-60	6.45	7.08	6.3	7.4	221	214	24.5	24.7
Lake 260 sediment	1-5	f	5	5	26-31								
Day 0 Initial Fish	1-5	m	5	5	1-5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	6-10*	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*Fish in tube # 10 appeared to be a male, not a female.

Table 29. Results from the *P. promelas* (FHM) embryo/larval test using elutriate samples prepared from the Canadian ELA Lakes 114 and 260 Sediments. Conducted 8/09/02. Test used 50 ml of test solution, 40 FHM eggs/replicate, MHRW control, 5 ng/l EE2 positive control and 2 ELA samples. Duration was 5 days. **Study #2-40.**

						pH		DO		Conductivity		Temp	
Conc.	**Rep #**	**% Sur**	**CV**	**t-test p value**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	98.8	1.1	n/a	1-5	8.35	8.08	8.4	8.0	327	335	24.1	24.7
Cont + DMSO	1-5	93.6	3.8	0.013	6-10	8.28	8.01	8.7	8.0	327	331	24.1	24.6
5 ng/l EE2	1-5	97.4	1.4	0.108	11-15	8.28	8.04	8.7	8.1	327	340	24.0	24.6
Lake 114	1-5	96.8	4.0	0.302	16-20	7.13	7.14	8.8	8.1	251	268	24.1	24.5
Lake 260	1-5	98.2	2.1	0.580	21-25	6.16	6.21	7.5	7.7	228	239	24.1	24.5

Bold/underline indicates sample determined to have survival statistically different from the control, t-test, alpha = 0.05.

Table 30. Results for the Adult FHM Gene Expression test with 2 ELA Lake samples. Conducted 10/27/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and samples from Lakes 239 and 260. Duration was 24 hours. **Study #2-56.**

						pH		DO		Conductivity		Temp	
Conc	**Rep#**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	1-5	7.98	8.09	7.9	7.6	315	364	24.1	24.7
MHRW	1-5	f	5	5	26-30								
Cnt + DMSO	1-5	m	5	5	6-10	7.96	7.66	8.0	7.6	312	343	24.3	24.5
Cnt + DMSO	1-5	f	5	5	31-35								
5 ng/l EE2	1-5	m	5	5	11-15	8.01	7.86	8.0	7.6	310	333	24.0	24.6
5 ng/l EE2	1-5	f	5	5	36-40								
Lake 239	1-5	m	5	5	16-20	6.80	7.50	9.2	7.4	28	52	24.5	25.1
Lake 239	1-5	f	5	4*	42-45								
Lake 260	1-5	m	5	5	21-25	6.78	7.52	9.4	7.5	22	60	24.6	24.9
Lake 260	1-5	f	5	5	46-50								
Day 0 Initial Fish	1-5	m	5	5	A	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	A	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*Female fish in rep # 1 dead. No tube 41.
A Refer to initial fish used in Study 2-55 for information on baseline fish data.

Table 31. Results for the Adult FHM Gene Expression test with 2 ELA Lake sediment samples Conducted 12/16/02. Treatments included MHRW control, MHRW + DMSO, 5 ng/l EE2 positive control sample and sediment samples from Lakes 239 and 260. Duration was 48H. **Study #2-64.**

						pH		DO		Conductivity		Temp	
Conc	**Rep #**	**sex**	**exp**	**live**	**Tube#**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	m	5	5	11-15	8.17	7.56	8.3	7.4	335	345	24.4	24.4
MHRW	1-5	f	5	4*	41-44								
Cont + DMSO	1-5	m	5	5	16-20	8.12	7.68	8.1	7.5	333	350	24.1	24.7
Cont + DMSO	1-5	f	5	5	46-50								
5 ng/l EE2	1-5	m	5	5	21-25	8.07	7.58	8.0	7.0	330	346	24.1	25.2
5 ng/l EE2	1-5	f	5	5	51-55								
Lake 239 sediment	1-5	m	5	5	31-35	7.23	5.86	8.4	6.9	312	238	24.0	24.7
Lake 239 sediment	1-5	f	5	5	61-65								
Lake 260 sediment	1-5	m	5	5	36-40	7.43	6.65	7.6	7.0	307	288	24.0	24.9
Lake 260 sediment	1-5	f	5	5	66-70								
Day 0 Initial Fish	1-5	m	5	5	1-5	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Day 0 Initial Fish	1-5	f	5	5	6-10	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*Female fish in rep # 5 dead. No tube 45.

Table 32. Results from the *P. promelas* (FHM) embryo/larval test using elutriate samples prepared from the Canadian ELA Lakes 239 and 260 Sediments. Conducted 12/18/02. Test used 50 ml of test solution, 40 FHM eggs/replicate, MHRW control, 10 ng/l EE2 positive control and 2 ELA samples. Duration was 5 days. **Study #2-65.**

						pH		DO		Conductivity		Temp	
Conc.	**Rep #**	**% Sur**	**CV**	**t-test pvalue**	**Tube #**	**I**	**F**	**I**	**F**	**I**	**F**	**I**	**F**
MHRW	1-5	97.0	2.2	n/a	1-5	7.74	7.94	8.2	7.7	324	347	25.1	24.3
Cont + DMSO	1-5	95.5	4.7	0.516	6-10	7.89	8.04	8.2	7.4	321	350	25.1	24.4
10 ng/l EE2	1-5	97.0	2.8	1.000	11-15	7.91	8.00	8.2	7.5	321	342	25.0	24.4
Lake 239	1-5	98.0	2.8	0.548	16-20	7.27	7.64	7.6	7.1	231	240	25.1	24.8
Lake 260	1-5	94.0	4.0	0.160	21-25	6.55	7.48	8.2	7.3	224	238	25.1	24.5

Bold/underline indicates sample determined to have survival statistically different from the control, t-test, alpha = 0.05.

Table 33. Summary of *P. promelas* survival data for Canadian ELA Elutriate toxicity tests. Elutriates were prepared using ELA sediments and MHRW. FHM fry were 36 to 48 hours old at the start of the test. Test duration was 48-H, with a test solution renewal at 24-H. CB indicates sediment sample collected at the Center Buoy of the Lake. SS indicates sediment sample collected from the near shore surface sediment. **Study # 3-37.**

SITE ID	**Collection Date**	**Mean % Survl**	**S.D.**	**C.V. %**	**t-test**
MHRW + DMSO	n/a	99.5	0.0112	1.1	n/a
5 ng/l EE2	n/a	99.5	0.0112	1.1	1
Lake 114 CB	9/21/03	99.5	0.0112	1.1	1
Lake 260 SS	9/21/03	96.7	0.0271	2.8	0.062
Lake 260 CB*	9/21/03	8.5	0.163	191.8	0.008

*Sample determined to be statistically different from MHRW for survival using t-test, alpha = 0.05.

TABLE 34. Initial routine chemistries for *P. promelas* tests. **Study # 3-43.**

	pH		D.O. (ppm)		Conductivity (S)		Temp (°C)	
sxs	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**
(-) Cnt.	7.96	8.30	8.7	8.6	332	336	24.1	24.6
(+) Cnt	7.91	8.27	8.5	8.5	332	336	24.1	24.6
S+0 ug/l	7.79	8.18	8.8	8.0	325	320	24.1	26.2
S+1 ug/l	7.79	8.18	8.8	8.3	336	329	24.1	25.6
S+50 ug/l	7.80	8.16	8.8	8.0	334	330	24.1	26.2
S+500 ug/l	7.81	8.17	8.7	7.9	336	330	24.1	26.2
239+0 ug/l	5.66	6.19	7.8	8.6	179	175	24.1	25.8
239+1 ug/l	5.57	6.01	7.3	8.6	176	173	24.1	25.8
239+50 ug/l	5.62	5.98	7.1	8.6	175	171	24.1	25.7
239+500 ug/l	5.57	5.95	7.6	8.7	174	170	24.1	25.3
260+0 ug/l	5.37	5.86	7.1	8.1	173	166	24.1	25.2
260+1 ug/l	5.31	5.66	7.0	8.0	173	170	24.1	25.1
260+50 ug/l	5.33	5.67	6.6	7.8	174	170	24.1	25.3
260+500 ug/l	5.27	5.64	6.8	7.7	174	171	24.1	25.3

TABLE 35. Final routine chemistries from *P. promelas* toxicity tests. **Study # 3-43.**

	pH		D.O. (ppm)		Conductivity (S)		Temp (°C)	
sxs	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**	**24 hr**	**48 hr**
(-) Cnt.	8.07	8.00	7.7	7.9	337	339	24.4	24.8
(+) Cnt	8.27	7.81	7.7	7.9	336	346	24.8	24.8
S +0 ug/l	8.18	8.12	7.8	7.8	329	336	24.8	24.6
S +1 ug/l	8.13	8.14	7.5	7.8	344	355	24.4	24.6
S +50 ug/l	8.10	8.10	7.7	7.8	333	345	24.7	25.0
S +500 ug/l	8.07	8.10	7.6	7.8	337	346	24.3	24.7
239+0 ug/l	7.29	7.29	7.4	8.2	186	192	24.8	24.9
239+1 ug/l	7.30	7.31	7.7	8.2	188	186	24.9	24.8
239+50 ug/l	7.28	7.32	7.9	8.2	176	188	24.6	24.4
239+500 ug/l	7.32	7.38	7.9	8.2	181	185	24.7	24.7
260+0 ug/l	6.78	6.87	8.0	8.2	175	181	24.7	24.7
260+1 ug/l	6.75	6.81	7.9	8.0	184	182	24.9	25.1
260+50 ug/l	6.79	6.67	7.6	8.0	170	187	24.7	24.9
260+500 ug/l	6.78	6.84	7.9	8.0	179	177	25.0	24.7

Table 36. Summary of *P. promelas* survival data for Canadian ELA Elutriate toxicity tests. Elutriates were prepared using ELA sediments and MHRW. FHM fry were 24 to 48 hours old at the start of the test. Test duration was 48-H, with a test solution renewal at 24 hours and a 25ºC test temperature. **Study # 3-43.**

SITE ID	Collection Date	Mean % Survl	S.D.	C.V. %
MHRW + DMSO	N/A	100.0	0	0
MHRW + 10 ng/l EE2	N/A	99.0	0.014	1.38
Sand + 0 DMSO	N/A	100.0	0	0
Sand + 1ug/l DMSO	N/A	100.0	0	0
Sand + 50 ug/l DMSO	N/A	100.0	0	0
Sand + 500 ug/l DMSO	N/A	99.5	0.011	1.12
239 + 0 DMSO	10/30/03	100.0	0	0
239 + 1 ug/l DMSO	10/30/03	99.0	0.014	1.38
239 + 50 ug/l DMSO	10/30/03	99.5	0.011	1.12
239 + 500 ug/l DMSO	10/30/03	100.0	0	0
260 + 0 DMSO	10/30/03	100.0	0	0
260 + 1 ug/l DMSO	10/30/03	100.0	0	0
260 + 50 ug/l DMSO ^	10/30/03	94.5	0.057	6.03
260 + 500 ug/l DMSO	10/30/03	100.0	0	0

^ Indicates this concentration originally contained no fry. Restarted this concentration the following day.