Examination of the Environmental Fate and Effects of POEA in Shallow Freshwater Ecosystems

By

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A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Abstract

Traditional herbicide formulations such as Roundup® contain the active ingredient glyphosate paired with the non-ionic surfactant polyethoxylated tallow amine (POEA). The impacts of POEA in aquatic environments are uncertain. In this study the environmental fate and effects of POEA was evaluated. A mesocosm field study confirmed that POEA dissipated rapidly from the water, but was persisted in the sediment; biological effects were negligible. In the laboratory, histological analysis of gills did not indicate negative effects on gill function in *Pimephales promelas* exposed to POEA. Proliferation of mucous cells in gills was significantly greater following 7 days of exposure. Liver histology appeared normal following exposures. Mean thiobarbituric reactive substances (TBARS) doubled in minnow livers exposed to 10 $\mu g.L^{-1}$ POEA for 7 days; however was not statistically significant. The present study indicates that POEA may persist in sediment and may influence benthic communities over the long term.

Acknowledgements

I would first like to express my sincere gratitude to my advisor, Dr. Gary Anderson, for the continuous support and motivation through my thesis project. His guidance and expert knowledge helped me through the laboratory component of my research and the writing of this thesis. I would also like to convey my appreciation to my co-advisor, Dr. Vince Palace, who initially got me involved in this project and provided me with the unique opportunity to conduct my field research at the Experimental Lakes Area. Besides my advisors, I would like to thank the rest of my thesis committee: Dr. Jason Treberg and Dr. Mark Hanson, for their insightful comments and technical support, but also for their leadership, which broadened the scope of my research.

I am grateful for the personal funding and support through the Government of Canada Research Affiliate Program, the Contaminants Advisory Group at Department of Fisheries and Oceans Canada, the University of Manitoba Faculty of Science, Faculty of Graduate Studies, Department of Biological Sciences, and the William O. and Erna Pruitt Graduate Bursary.

I'd also like to acknowledge the group that conducted the sample analysis at the University of Guelph Laboratory Services Division: Dr. Linda Lissemore, Jose Luis Rodriquez-Gil, Dr. Mark Hanson, and Dr. Keith Solomon. The staff that analyzed the samples at the Laboratory of Expertise for Aquatic Chemical Analysis: Andrew Ross and Xiangjun Liao. I'd also like to thank the staff and students at the Experimental Lakes Area and Freshwater Institute who helped me along the way: Suzanne Mittermuller, Kerry Wautier, Bruno Rosenburg, Alan Dupuis, Brad Park, Michelle Nicholson, Amy Gilbert, Jaycee Catt, Paul Fafard, and Kirstin Dangerfield.

Finally, a sincere thanks to my family and friends who put up with declined invitations and last minute cancelations throughout this time. Your understanding has allowed me to reach this milestone.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction and Literature Review

1.1. Introduction

Glyphosate is the most widely used herbicide in the world and is the active ingredient in commercial formulations such as Roundup®, Honcho®, Sting®, Alphee®, Azural® and Faena®. It is the second most commonly applied herbicide in the United States, with 38 – 43 x 10^6 kg used in agricultural, home, garden, forestry and wetlands (Donaldson et al., 2002). Its use is increasing with the emergence of genetically modified crops such as "Roundup Ready®" (Monsanto). Glyphosate normally constitutes 41% of the herbicide formulation by weight (Bradberry et al., 2004). It is a broad spectrum herbicide that competitively inhibits the enzyme enolpyruvylshikimate phosphate synthase, blocking the synthesis of the essential aromatic amino acids, tyrosine, tryptophan and phenylalanine (Giesy et al., 2000). In plants, these amino acids are used for the synthesis of proteins, which are necessary for growth and development (Petrie, 1943). Glyphosate is effective only as a post-emergent herbicide because it requires absorption through foliage in actively growing plants to be effective. Therefore to enhance foliar uptake, glyphosate is most often formulated along with the adjuvant polyethoxylated tallow amine (POEA).

1.1.1. POEA characterization

POEA is a non-ionic surfactant frequently added to glyphosate-based herbicides as a wetting agent (Table 1.1 & Figure 1.1). It generally constitutes less than 15% of the herbicide formulation by weight (Giesy et al., 2000). In the glyphosate-based herbicide Roundup®, POEA is assigned the trade name MON 0818, (Monsanto, 1990). POEA is formulated when 1 mol of

tallowamine is exothylated through the addition of 2 mol ethylene oxide (EU, 2008). The mass ratio between the oxide and tallowamine sections of the compound characterize POEA (Brausch & Smith., 2007) and ranges from 5:1 – 25:1 (Huntsman, 2005a, b). The solubility of POEA in water increases with increasing oxide:tallowamine ratio (Brausch & Smith, 2007).

POEA is an amphiphilic compound; meaning it has both a hydrophobic (not soluble in water) and hydrophilic group (soluble in water). The hydrophobicity originates from the "tallow" portion of the molecule; derived from animal fats (Solomon et al., 2003). Tallow contains a variety of fatty acids including oleic (37 - 43%), palmitic (24 - 32%), stearic (20 - 25%), myristic (3 - 6%), and linoleic (2 - 3%) acids as well as small amounts of cholesterol, arachidonic, elaidic, and vaccenic acids (Budavari, 1989). The non-ionic property of POEA is related to the hydrophilic group, which interacts with water at the ether oxygens of the polyethylene chains. Since herbicides are most commonly applied as an aqueous solution, POEA is able to disperse and adsorb to the waxy cuticle of the plant at the water interface. Here, the hydrophobic tails associate with the cuticle and the hydrophilic heads associate with the water molecules. The surfactant enhances the wetting of the plant cuticle surface, breaking the surface tension and allowing the active ingredient, glyphosate, to penetrate into the plant tissues (Bonn, 2013).

The degradation of POEA has not been studied explicitly and only a few studies involving similar compounds, such as alkylamine ethoxylates (ANEO), have been published from which we can extrapolate. Although degradation in the environment can occur abiotically or biotically, to my knowledge, only biodegradation of ANEOs has been investigated (Krogh et al., 2003). The biodegradation pathways of POEA have been proposed using a *Pseudomonas* species. The catabolic pathway begins with central fission, followed by degradation of the two intermediates; an aldehyde and an ethoxylated secondary amine (Van Ginkel et al., 1993) with the final

products being CO_2 and water.

Banduhn and Frazier (1974) estimated the half-life of POEA in natural waters containing suspended sediment to be 3 - 4 weeks. Based on this, Giesy et al. 2000 in their conservative risk assessment, assumed 21 – 42 days for 50% dissipation. In soils, Marvel et al. (1974) reported a half-life of < 7 days, and Giesy et al. (2000) used a conservative estimate of 7 – 14 days in their risk assessment. No values are available for aquatic sediments.

1.2. Exposure Pathways

Glyphosate formulations may be applied by directed foliar, ground broadcast foliar, or aerial methods (SERA, 2003). Isopropaline (IPA) salt of glyphosate is the form found in Roundup® formulations, but glyphosate acid is what binds at the target site of the plant. The acid equivalent (a.e.) is used when the concentrations are calculated from the IPA salt concentrations.

POEA has the potential to enter aquatic systems by runoff, incidental overspray or offsite drift from aerial applications. The environmental fate of POEA has not been adequately modeled in freshwaters and their underlying sediments although it is generally thought that POEA, like glyphosate, partitions quickly to sediments (Giesy et al., 2000; Wang et al., 2005).

The United States Environmental Protection Agency (USEPA) modeled an experimental aquatic exposure (USEPA, 2007). The USEPA assumed that glyphosate-based herbicides are typically applied at 2.24 kg acid equivalent (a.e.).ha^{-1} but that a maximum of 7.85 kg (a.e.).ha^{-1} was also used to derive worst case scenario exposures (equivalent to 2.99 to 10.46 kg active ingredient (a.i.).ha^{-1}). It was assumed that approximately 8% of the glyphosate formulation by weight was POEA, so at these application rates there would be 0.58 and 2.04 $kg.ha^{-1}$ POEA applied on

average and as a maximum, respectively (USEPA, 2007). The USEPA modeled the remaining percentages of POEA in both a pond and a stream at varying distances from an application of glyphosate assuming a high boom or aerial application (stream flows and water volumes were not provided). Using the modeled environmental exposure data and an assembly of selected toxicity values for POEA, risk quotients for expected concentrations against lethality curves were derived.

The USEPA concluded that there was little cause for concern except at the distance of 0 m (i.e. right at the point of application). However, one of the overlying assumptions of the modeled process was that no leaching or runoff occurred. This assumption is in general agreement with the literature in assuming that POEA does not leach from soils/sediments because of its strong sorption characteristics (Giesy et al., 2000). In fact, the leaching potential for POEA has been quantified as relatively small (i.e. less than 2% of application) (Giesy et al., 2000).

Using the example of glyphosate, which is also determined to bind rapidly to sediments, results from ground water monitoring in Sweden found glyphosate concentrations from 0.1 - 1.0 $\mu g.L^{-1}$ with the greatest detected value of 13 $\mu g.L^{-1}$ (Pettersson et al., 2006). Furthermore, values between 0.1 - 0.7 $mg.L^{-1}$ Roundup® have been recorded in streams near soybean cultivation in Argentina (Perusso et al., 2008). Concentrations of glyphosate have been detected in agricultural ponds at 0.09 - 1.7 mg glyphosate (a.e.).L^{-1} in pond water and 0.26 - 19 mg (a.e.).L^{-1} in pond sediment (Giesy et al., 2000). All these data suggest that, despite its strong sorption to sediments, glyphosate can find its way into ground and surface waters. Regrettably, analytical challenges such as selecting for polyethoxylated alkyl amines in the sample that are representative of the entire exposure have precluded similar analysis for POEA. However, using estimates of 15% POEA in glyphosate formulations and assuming that the compound partitions similarly to

glyphosate, the residues above correspond to a range in concentrations for POEA in ground and receiving waters from 0.03 - 622 $\mu g.L^{-1}$, and 0.09 - 6.95 $mg.L^{-1}$ in aquatic sediments.

To my knowledge, only a single study has considered the fate of POEA in microcosms. Wang et al. (2005) added MON 0818 (Monsanto) at 8 $mg.L^{-1}$ (comprised of 75% POEA) to 72 L aquaria with or without 3 cm of two different natural sediments containing either 1.5 or 3% total organic carbon (TOC). Water samples were collected at 2, 6, 24, 48, 72 and 96 hrs post addition and used to measure MON 0818, as well as to perform toxicity tests using the water flea, *Daphnia magna*, as the test organism. Concentrations of MON 0818 persisted throughout the exposures in aquaria without sediment. Mortality was also greater among *D. magna* in the aquaria with no sediment. The amount of time required for 50% of the initial concentration of MON 0818 to dissipate (DT50) was 13 and 18 hrs in the aquaria containing 1.5 and 3% TOC sediments, respectively. The authors recommended additional studies to determine concentrations of MON 0818 in sediments, not just in the overlying water as they reported. Additionally, they commented that their experimental design did not allow them to separate sorption of POEA to the sediment from microbial degradation. Specific distinction between sorption to clay or organic carbon components of the sediments was also not determined.

1.3. Toxicity of POEA and Formulations Containing POEA

The main toxicological effect of POEA is disruption of cell membranes on the respiratory surfaces of exposed organisms (Brausch et al., 2007). POEA containing Roundup® also affects the transmembrane potential of mitochondria and uncouples the electron transport system at a concentration of 15 mM (Peixoto, 2005). Whereas the bulk of toxicological studies have

investigated glyphosate toxicity, some information can be gleaned from experiments in which formulations containing POEA have been evaluated alongside formulations without the adjuvant.

1.3.1. POEA effect on invertebrates

Studies focusing on *D. magna* provide the most complete dataset, in regard to POEA toxicity. Brausch et al. (2007) exposed one day old *D. magna* for 48 hrs to various concentrations of three different POEA formulations that varied in their ratios of oxide:tallowamine (5:1, 10:1, and 15:1). Test concentrations were 0.1, 1, 10, 100, 500, 1000, or 10000 $\mu g.L^{-1}$. Survivors at the end of the 48 hr exposure were removed for eyespot to carapace end length measurements. The 10:1 formulation was most toxic (LC50 = 97 $\mu g.L^{-1}$) followed by 5:1 (LC50 = 176.4 $\mu g.L^{-1}$) and 15:1 (LC50 = 849.4 $\mu g.L^{-1}$). Growth was inhibited by the greatest concentration of all formulations, but also for the most toxic (10:1) formulation at concentrations as low as 100 $\mu g.L^{-1}$ POEA. Inhibition of growth is an important endpoint for *D. magna*, because adults become sexually mature based on size and not age (Schwartz, 1984). The same authors tested the effects of POEA on fairy shrimp (*Thamnocephalus platyurus)* nauplii and found that they were about 400 times more sensitive than *D. magna*. However, in this case it was a 15:1 formulation that was most toxic (15: 1 > 10:1 > 5:1) (Brausch et al., 2007).

Giesy et al. (2000) reviewed the literature for POEA acute toxicity and tabulated EC50 values. The invertebrate species reviewed included *Chironomus plumosus, D. pulex,* and *D. magna*. The EC50/LC50 values ranged from 2 - 13 $mg.L^{-1}$. The data appear to agree well with the LC50 derived in the microcosm experiments of Wang et al. (2005), described above (48 hr LC50 = 2.9 $mg.L^{-1}$). Giesy et al. (2000) estimated a chronic toxicity value of 0.1 $mg.L^{-1}$ POEA based on the available data, citing a lack of existing data for POEA in invertebrates.

Tsui and Chu (2003) examined toxicity of Roundup®, POEA, glyphosate acid, and an IPA salt of glyphosate on selected crustaceans, protozoans, algae, and bacteria. They determined LC50 values for POEA of 0.57 – 1.0 $mg.L^{-1}$ for crustaceans, approximately 5 $mg.L^{-1}$ for protozoans, 3.5 - 4 $mg.L^{-1}$ for algae, and >10 $mg.L^{-1}$ for bacteria. In addition, they examined the effects of temperature, pH, suspended particles, and food availability on the toxicity of these compounds to crustaceans. Results indicated that while temperature and food ration had little effect, an alkaline pH and the presence of suspended particles increased toxicity. The effect of pH was likely due to the non-ionic properties of surfactants in alkaline conditions (pH >8). They speculated that particles may have absorbed POEA and/or glyphosate and that the filter feeding crustaceans assimilated these loaded particles.

The effects of low (11 mg glyphosate + 3.75 mg POEA) and high (22 mg glyphosate + 7.5 mg POEA) environmental concentrations of herbicides were examined in a chronic 50 day study using freshwater crayfish (*Cherax quadricarinatus*) (Frontera et al., 2011). Growth, energy storage as glycogen, and consumption of protein, lipid, and oxygen were examined in each of the groups. Growth and oxygen consumption was lower in all groups and POEA exposure resulted in lower lipids in muscle and hemolymph, while glyphosate appeared to affect glycogen reserves to a greater degree. The authors speculate that the two may act synergistically by depressing mitochondrial complexes affecting oxidative phosphorylation.

1.3.2. POEA effects on amphibians

POEA has been shown to be acutely toxic to amphibians (Diamond & Durkin 1977; Howe et al., 2004; Relyea, 2005) and about 700 times more toxic than glyphosate itself (Perkins et al., 2000). Howe et al. (2004) exposed four species of frogs to several formulations of glyphosate, with and

without POEA, and found that the formulations with POEA were more toxic than formulations with a blend or unknown adjuvants. *R. clamitans* was nine times more sensitive to Roundup Original® (96 hr LD50 = 6.6 $mg.L^{-1}$) than Roundup Transorb®, Glyfos AU®, Roundup Biactive®, Touchdown®, and Glyfos BIO® (96 hr LD50 > 57.7 $mg.L^{-1}$). In these experiments, exposure to POEA resulted in greater time to metamorphose, increased frequency of damage to the tail (a region of high metabolic activity and cell division), and reduced size at metamorphosis. Regression of the tail in metamorphosing frogs is mediated by the thyroid system, and in frogs exposed to some formulations of glyphosate (Roundup Original® and Roundup Transorb®), expression of thyroid hormone β receptor was induced, but POEA alone did not affect expression. The authors note that sex steroids have the potential to alter sexual characteristics by interfering with the thyroid axis and suggest that this indirect mechanism warrants further investigation.

Edge et al. (2014) exposed *Lithobates sylvaticus* egg masses to 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg glyphosate (a.e.).L^{-1} from Roundup Weed and Grass Control® and Roundup WeatherMax®. Specimens were collected from four different North American wetlands sites and ran as separate experiments. The 96 hr LC50 values for both formulations varied among the populations (Roundup Weed and Grass Control® = 0.09 - 1.10 mg glyphosate (a.e.).L^{-1}; Roundup WeatherMax® = 4.94 - 8.26 mg glyphosate (a.e.).L^{-1}). The authors speculate increased sensitivity of *L. sylvaticus* larvae to Roundup Weed and Grass Control® as compared to Roundup WeatherMax® is likely due to variances in surfactants or adjuvants among formulations.

Reylea (2005) conducted a mesocosm study to examine the effects of Roundup® formulation (glyphosate with POEA) on organisms selected to approximate natural aquatic systems that may

potentially be impacted by herbicide overspray. When Roundup® was applied at what would constitute maximum rates of use and a 100% overspray condition onto the surface of the mesocosms (3.8 $mg.L^{-1}$), species richness was significantly affected. This was primarily the result of almost complete extirpation of large herbivores (tadpoles and snails) with little or no effect on periphyton. In additional experiments, 96 hr LC50 values for six species of North American tadpoles ranged between 0.5 and 2.5 $mg.L^{-1}$ (Reylea, 2005).

Mann and Bidwell (1999) determined acute toxicity of several formulations of glyphosate to four species of Australian frogs. They concluded that POEA was the source of the greatest toxicity. Forty-eight hour LC50 values ranged from 2.9 - 11.6 mg glyphosate (a.e.).L^{-1}. Adults and new metamorphs were less sensitive than tadpoles; and among tadpoles, larger body size tended to reduce toxicity. The authors note that amphibian habitat is complex and that acute toxicity testing may not be representative of real world effects. They urge microcosm or mesocosm testing as a useful method to evaluate potential glyphosate formulation effects.

1.3.3. POEA effects on fishes

While there are many studies examining the acute toxicity of glyphosate and POEA to fish, there are few studies that examine the effects of chronic exposure to either compound (Jiraungkoorskul et al., 2003). POEA, like other surfactants, may interfere with gill morphology, cause lysis of gill epithelial cells, as well as result in protein solubilization (Partearroyo et al., 1991). Folmar et al. (1979) conducted a series of experiments examining several aspects of glyphosate formulation and surfactant effects. Despite being completed more than 30 years ago, this study is still highly cited and the results remain relevant with respect to acute toxicity. Acute toxicity of glyphosate, IPA salt of glyphosate, Roundup®, and MON 0818 was determined for

four freshwater invertebrate species (*D. magna, Gammarus pseudolimnaeus*, *C. plumosus*, *Ephemerella walkeri*) and four freshwater fish species (*Salmo gairdneri, Pimephales promelas, Channa punctatus, Lepomis macrochirus).* The 96 hr LC50s of MON 0818 was 1.0 $mg.L^{-1}$ for *P. promelas*, 2 $mg.L^{-1}$ for *S. gairdneri*, 3 $mg.L^{-1}$ for *L. macrochirus* and 13 $mg.L^{-1}$ for *C. punctatus*. In a life stage test, eyed eggs were least sensitive, fry increasing in their sensitivity, and larger fingerlings becoming less sensitive. Lower hatch rates were documented among *S. gairdneri* eggs exposed to 5 mg Roundup®.L^{-1} and reduced survival among eggs and fry at 10 and 20 $mg.L^{-1}$, respectively. Higher temperatures increased toxicity of Roundup® to *S. gairdneri* and *L. macrochirus*, with double the toxicity at 17°C compared with 7°C for *S. gairdneri*. Altering pH had minor impacts, with an increase in Roundup® and MON 0818 toxicity when pH was raised from 6.5 to 7.5, but no further effect up to pH 9.5. Finally, adult *S. gairdneri* exposed for 12 hrs at spawning time and then allowed to depurate for 30 days, had no negatively impacted gonadal development. While much of the data additional to the acute toxicity studies was conducted with Roundup® alone, the authors point out that MON 0818 appeared to comprise most of the toxicity.

In contrast to the results of Folmar et al. (1979), Mitchell et al. (1987) found little significant difference in the acute toxicity of Roundup® or the aquatic herbicide Rodeo (tested with the surfactant ortho X-77) on *S. gairdneri*, *Onchorhynchus tschawytscha*, and *Onchorhynchus kisutch*. Furthermore, they found that altering pH or water hardness had little effect on 96 hr LC50 values in all of these species. Wan et al. (1989) compared toxicity of Roundup®, glyphosate and MON 0818 in five species of Salmonids: *O. tschawytscha, Onchorhynchus gorbuscha, Onchorhynchus keta, S. gairdneri*. They confirmed that MON 0818 was the most toxic portion of the formulation, but also noted wide variations in 96 hr LC50s between the five

species with additional effects on toxicity introduced by varying pH and hardness of water.

Hued et al. (2012) exposed *Jenynsia multidentata*, a fish species native to Argentina, to 0.5 $mg.L^{-1}$ of Roundup® for 7 or 28 days and examined reproductive behaviour as well as gill and liver histopathology. The 96 hr LC50 in this species was determined to be 19 $mg.L^{-1}$, making it moderately sensitive. Male reproductive behaviour was inhibited after exposure at both time points and both gill and liver pathologies responded dose-dependently. In gill, epithelial cell lifting, hypertrophy and hyperplasia were documented, which is consistent with respiratory surface impacts from glyphosate formulations. The authors speculate that this may be an adaptive mechanism to increase diffusion distances across the respiratory surface and to reduce the uptake of contaminant. In liver, hydropic degeneration (epithelial cells absorb lots of water), blood sinusoidal dilation, foci of leukocyte infiltration, and necrosis were reported.

Szarek et al. (2000) described intracellular changes in liver of carp exposed for a short-term (0.5 - 1 hr) to relatively high concentrations of Roundup® (205 or 410 $mg.L^{-1}$). Effects included the appearance of myelin-like structures in hepatocytes, swollen mitochondrion with loss of cristae, enlarged Golgi and rough endoplasmic reticulum canals, as well as reduced glycogen. It should be noted that most fish from these studies died, and histopathology was investigated in survivors. Histopathological changes were also noted in another study in which carp were exposed to sub-acute levels of glyphosate (2.5, 5 or 10 $mg.L^{-1}$) (Neskovic et al., 1996). Gill effects included hyperplasia, subepithelial edema, and chloride cell proliferation, which may have been related to acid-base balance disturbance. Some biochemical disturbances were also documented, most notably an increase in hepatic alkaline phosphatase at all exposure concentrations (indicative of bile duct occlusion). It is uncertain if the commercial glyphosate formulation in this experiment included a POEA based surfactant.

Ayoola (2008) also noted histopathological changes in juvenile Nile tilapia exposed for 96 hrs to glyphosate. They determined the 96 hr LC50 to be 1.05 $mg.L^{-1}$. Cell proliferation, lamellar fusion, and epithelial lifting was evident at exposures above 9 $mg.L^{-1}$. In kidney, tubular epithelial pyknosis (condensation of chromatin in the nucleus of a dying cell) and hyaline droplet (reabsorbed proteins) formations were noted at >30 $mg.L^{-1}$ glyphosate; while in liver, vacuolation and necrosis was documented >9 $mg.L^{-1}$. It appears that a surfactant was included in this study based on the percent active ingredient noted by the authors (48%, which is standard for Roundup® formulations that also contain POEA as an adjuvant).

Using short-term exposures of 96 hrs, Glusczak et al. (2006) examined effects of Roundup® (0, 3, 6, 10, or 20 $mg.L^{-1}$) on several biochemical parameters in *Leporinus obtuscidens*. Acetylcholinesterase activity (AChE) was lower in the brain of all groups of exposed fish although skeletal muscle AChE was not affected. They note that this effect could impact prey capture and predator avoidance because of its effect on motor neuron function. In addition, liver glucose and glycogen increased while the same parameters declined in skeletal muscle at all exposures. This may indicate a general stress response as energy is mobilized to deal with contaminant stress. In support of this, lactate and protein declined in liver, and ammonia concentrations increased at all exposure levels. Several hematological parameters were also negatively impacted in exposed fish, including haematocrit (volume percentage of erythrocytes in blood), plasma protein, hemoglobin, and erythrocyte counts. Leukocyte counts were unaffected. It should be noted that the 96 hr LC50 for this species was >100 $mg.L^{-1}$ and so the exposure levels in this experiment were not lethal. In a similar follow up study, the same author examined effects following 96 hr exposures to 0.2 or 0.4 $mg.L^{-1}$ of Roundup® in *Rhamdia quelen* (Glusczak et al., 2007). Similar effects on glycogen in liver and muscle, as well as brain

AChE were noted at both exposures. Responses of the oxidative stress parameters, catalase and TBARS, were not consistently affected by Roundup®.

Jiraungkoorskul et al. (2003) noted histopathological effects in gills (thickened epithelium, clubbing of lamellae), liver (swollen hepatocytes, vacuole formation) and kidney (proximal tubule epithelial swelling, necrosis, pyknosis) of juvenile *Oreochromis niloticus* (15 - 20 g) chronically exposed (1 - 3 months) to 5 or 15 $mg.L^{-1}$ Roundup®. These authors also noted an increase in plasma aminotransferase activities of alanine and aspartate transaminase (ALT and AST). Since they are measured in plasma, these elevated enzyme activities, normally isolated to hepatic cells, may be indicative of liver damage. Langiano and Martinez (2008) reported elevated glucose and histopathological effects in liver of the Neotropical fish, *Prochilodus lineatus*, exposed to Roundup®. As 96 hr LC50 was derived (13.7 $g.L^{-1}$), and transient effects in plasma electrolytes were noted in fish exposed to 7.5 or 10 $mg.L^{-1}$ for short-term exposures (6, 24, or 96 hrs). In brief, it appears variable toxicity data are reported that appear to be largely dependent on formulations used.

1.4. Objectives and Hypotheses

It is important to note that even among the relatively recent chronic studies discussed above, there is a lack of acknowledgement that waterborne exposures alone are not environmentally relevant. That is, without incorporating sediment in the tests, the waterborne exposures represent longer than expected exposure due to POEA's high affinity to sediments.

My thesis had two specific objectives:

1) Determine the amount of time required for 50% of POEA to dissipate (DT50) in shallow freshwater lakes following incidental spray during aerial applications of glyphosate-based herbicides used by the agriculture and forestry industries.

2) Characterize the effects of short-term POEA exposure on cellular function and influence on select enzymatic reactions in fathead minnows (*Pimephales promelas*).

In an effort to identify the environmental fate of POEA, a mesocosm study was conducted at the Experimental Lakes Area (ELA), Ontario, Canada. The DT50 of POEA in the water column, bound to suspended particulates, and in aquatic sediments was examined. Two mesocosm designs (open-bottom: open to the sediments, and closed-bottom: partitioned from the sediments) were utilized to test if the DT50 of POEA (dosing at 10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$) in water is reduced with the presence of underlying sediments in a natural setting, as Wang et al. (2005) demonstrated in the laboratory. The presence of suspended particulates in both designs was expected to influence the DT50 of POEA, but the presence of POEA in the water of closed-bottom designs was predicted to persist relative to open-bottom designs.

The toxicity of POEA to fish was tested in a laboratory setting. Although there were no sediments added to aquaria, the same POEA treatment concentrations tested in the ELA mesocosm study were used. Fish were exposed for 2 or 7 days. Histological lesions in gills and liver, change in brachial Na^+/K^+-ATPase activities, and hepatic lipid peroxidation were investigated. Based on the results of a pilot study conducted at the ELA in addition to the mesocosm fate study, fish exposed to 100 $\mu g.L^{-1}$ POEA for 2 days were expected to show the largest reduction in the portion of gills available for gas exchange (PAGE), and to recover to normal morphology by the seventh day of exposure. Proliferation of mucous cells in the gills of

fish exposed to 100 µg.L^{-1} POEA was predicted to be the most severe (Pereira et al., 2012). Swelling of hepatocytes in fish was expected to be minimal due to the rapid dissipation of POEA and the successful function of mechanisms of protection (PAGE, proliferation of mucous cells) in the exterior organs (gills and skin). Any occurrence of hepatocyte swelling was expected to be greatest in the liver of fish exposed to 100 µg.L^{-1} POEA for 7 days (Hued et al., 2012). Inhibition of the ion transport enzyme, Na^+/K^+-ATPase was predicted to be greatest in the gills of fish exposed to 100 µg.L^{-1} POEA for 7 days (Haya et al., 1983). An elevation in TBARS, from damage due to oxidative stress, induced by lipid peroxidation was anticipated in liver of fish exposed to 100 µg.L^{-1} POEA for 7 days (Li et al., 2003; Liu et al., 2008).

Table 1.1. Physical and chemical properties of POEA

Characteristic	Information
Synonyms[a]	• Polyoxyethylated tallowamine, Polyoxyethylene tallowamine •MON 818 •Ethoxylated tallow alkyl amines •Tallow amine ethoxylate •Polyoxyethylene tallow amines •Ethomeen T, Ethomeen T/15 •Chemeen T series •Frigate, Frigate LO-dose •Katapol 25CWS, Katapol PN-430, Katapol PN-730, •Trymeen TAM series
CAS No.	61791-26-2 [b]
Chemical Formula	$R\text{-}N(CH_2CH_2O)H_m(CH_2CH_2O)H_n$ [b]
Molecular weight	Varies based on polyethylene chain length
Colour	Amber-yellow [b]
Physical state	Liquid [b]
Melting point	12°C [b]
Density (25°C)	1.02 kg/L [c]
Viscosity (37.78°C)[c]	96 cSt [c]
Log K_{oc} (silt loam, silt clay loam, and sandy loam)	2500 - 9600 [d]

[a] http://www.chemnet.com/cas/es/61791-26-2
[b] http://chemicalland21.com/specialtychem/perchem/ETHOXYLATED%20TALLOW%20AMINE.htm
[c] http://www.anshulindia.com/pdfs/AGNIQUE_TAM-15.pdf
[d] *Wang et al. 2005*

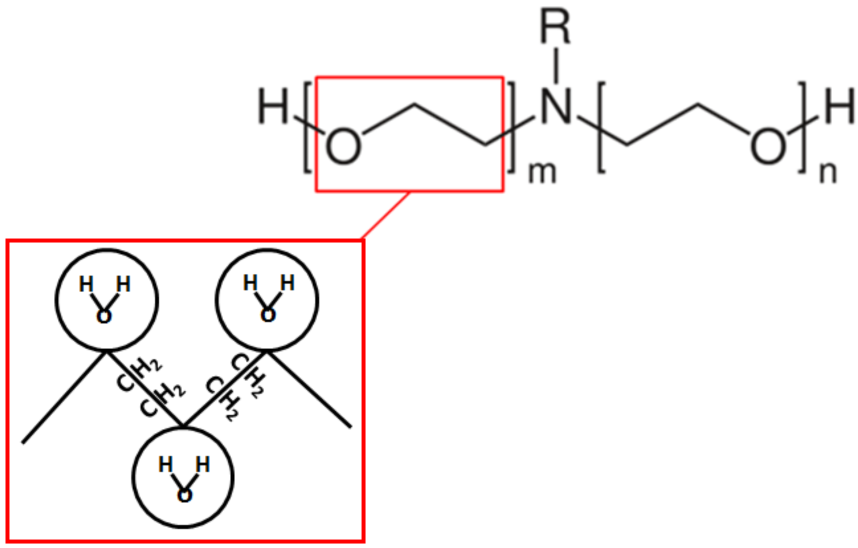

Figure 1.1. The chemical structure of POEA. Expanded view: hydrophilic interaction of water molecules at the ether oxygens of the polyethylene chain.

Chapter 2: ELA Mesocosm Study of the Environmental Fate of POEA

2.1. Introduction

POEA is a non-ionic surfactant frequently added to glyphosate-based herbicides as a wetting agent. It generally constitutes less than 15% of the herbicide formulation by weight (Giesy et al., 2000). Glyphosate is effective only as a post emergent herbicide because it requires absorption through foliage in actively growing plants to be effective. Therefore, to enhance foliar uptake, glyphosate is most often formulated along with the adjuvant POEA. One study that has considered the fate of POEA in a semi-natural setting, determined different half-lives of MON 0818 (Monsanto) in the water column of aquaria with and without sediment (Wang et al., 2005); furthermore, half-lives varied based on the composition of the sediments included. The authors recommended additional studies to determine concentrations of MON 0818 in sediments and not just in the overlying water as they reported. Giesy et al. (2000) reviewed the available data and described microbial degradation as the primary route of POEA removal from soils. They performed a Tier 1 (conservative worst case scenario) risk assessment to determine a half-life range based on model generated, maximum chronic exposures in aquatic systems, and on the mineralization of ^{14}C labelled POEA to CO_2 (Banduhn and Frazier, 1974 as reviewed by Giesy et al., 2000).

In the literature, there is insufficient information on the toxicity and environmental concentrations of POEA alone. Specifically, the environmental fate of POEA has not been sufficiently demonstrated in freshwaters and their underlying sediments. This gap at the time of this study, is partly due to the lack of a published method for POEA analysis. The current

assumption is that POEA does not leach from soils or enter aquatic systems via runoff as modeled in a USEPA report (2007).

Also, in the risk assessment of Giesy et al. (2000), model derived conclusions were used due to the lack of fate data for POEA in a natural setting. Howe et al. (2004) noted that data on environmental levels and persistence of POEA after field applications is lacking but that these data are required for a complete assessment of the acute and chronic toxicity of glyphosate formulations. Additionally, these authors note that because POEA adheres to particles that organisms may feed on, exposure may be higher than expected when only waterborne exposures are considered.

In support, a study conducted by Tsui and Chu (2003), indicated that while temperature and food ration had little effect, an alkaline pH and the presence of suspended particles (or high total suspended solids (TSS)) increased toxicity of POEA. The fate of POEA in sediments and the toxicity to benthic organisms are poorly understood. These data gaps have been identified as a critical need for the Ecological Risk Assessment process by Health Canada's Pest Management Regulatory Agency (PMRA).

In an effort to fill these data gaps, a mesocosm study was performed at the Experimental Lakes Area, Ontario, Canada to examine the environmental fate of POEA in shallow freshwater ecosystems. The objectives were as follows:

- To determine the DT50 of a one-time, environmentally relevant POEA treatment in the water column, bound to suspended particulates, and in aquatic sediments.
- To monitor the influence of aquatic sediment on the DT50 of POEA.
- To monitor the influence of TSS and TOC on the DT50 of POEA in the water column.
- To monitor the effects of POEA on aquatic biota; zooplankton and forage fish

2.2. Materials and Methods

2.2.1. Surfactant

POEA is characterized in Section 1.1.1. The POEA for this experiment was acquired from Monsanto Canada Inc. in Winnipeg, MB as 71% POEA under the trade name MON 0818.

2.2.2. Site description

The study was conducted in Lake 114 (L114) at the ELA aquatic research facility (N49°40.254' W93°45.615') (Figure 2.1). The facility is comprised of 58 lakes, which have been designated for aquatic research since 1967. L114 exhibits all the key characteristics for adequately studying POEA in a natural shallow freshwater system - high total organic carbon, areas of 1 meter in depth, and rich with forage fish (Solomon et al., 2003).

2.2.3. Enclosures

Mesocosms, also known as limnocorrals, are plastic enclosures designed for aquatic research (Figure 2.2). They are a widely accepted methodology to simulate near-natural conditions while reducing laboratory limitations (Bloesch et al., 1988). Each of the nine mesocosms were 2 m diameter and were comprised of an octagonal foam collar with an attached circular, impermeable, polyethylene curtain extending 1 m in depth (Currie Industries Ltd, Winnipeg, MB, Canada). The estimated volume for each mesocosm was 3140 L. Six of the enclosures were open to the sediments while three were closed at the bottom, essentially forming a bag. Assembly of the mesocosms took place on site, the day prior to deployment. PVC pipe was threaded through loops that were manufactured at the top, middle and bottom of the curtains. The

top most PVC pipe was attached to the foam collar using zip ties and sideline (braided multifilament polypropylene rope).

Prior to deployment, the placement of each mesocosm was determined using a bathymetric map of L114 to locate areas of 1 m in depth. A Hydrolab instrument (model: Quantra, serial#: QD 00106) was used from the boat to confirm the exact depth. If satisfactory, a cinder block was then attached to a float with sideline and deployed as a placement marker. This was repeated for each mesocosm.

The assembled mesocosms were transported individually by boat to each marker. The float was removed and the cinder block tied to the collar of the mesocosm. Once all mesocosms were deployed, a SCUBA diver was required to secure the curtains to the bottom of the lake using sandbags. Approximately 26 sandbags were set down along the skirt of each mesocosm to secure its position and to isolate the area from the surrounding lake. The soft sediment in L114 allowed the sandbags to sink in deeply and minimized the risk of diffusion through the sediments. An additional three cinder blocks were evenly spaced around the collar and attached with sideline. They were extended outward and anchored into the sediment in order to minimize horizontal sway. Closed-bottom designs were pumped full with water from the surrounding lake.

2.2.4. Pre-treatment data collection

Nine mesocosms were deployed near the outflow at a shallow water location (1 m depth) in L114 on May 16, 2012. The experimental design consisted of three treatments based on a previously published modelled risk assessment (Geisy et al., 2000). Each treatment was applied in duplicate to open-bottom mesocosm designs. (1) Open-bottom, control (no POEA added; Control A and B), (2) Open-bottom, low (treated with POEA at 10 $\mu g.L^{-1}$; Low A and B), (3) Open-bottom,

high (treated with POEA at 100 µg.L^{-1}; High A and B). In the closed-bottom designs the three treatments where applied with no replicates. (1) Closed-bottom, control (no POEA added; Control C), (2) Closed-bottom, low (treated with POEA at 10 µg.L^{-1}; Low C), (3) Closed-bottom, high (treated with POEA at 100 µg.L^{-1}; High C).

Pre-treatment sampling of water and suspended particulates was conducted on July 23, 2012, one day prior to dosing with the POEA. Pre-treatment sediment cores were taken on July 18, 2012 to allow for sufficient settling time prior to dosing. Samples of water and suspended particulates were also taken from the surrounding lake as reference.

2.2.4.1. Weather monitoring

Meteorological data including, air and water temperature, precipitation, and wind speed and direction was provided by Ken Beaty, ELA Hydrologist. The data was collected by the 2012 ELA hydrology field team, Neil Fogg and Amy Gilbert, under the supervision of Ken Beaty. All data, with the exception of water temperature, were collected at the ELA Meteorological Station located approximately 2 km from the experimental mesocosm site in L114. Air and water temperature (at surface), and wind speed and direction were collected hourly. Total precipitation was recorded every 24 hrs.

2.2.4.2. Nutrients

Pre-treatment water samples for nutrient analysis were collected on June 11, 2012 at 0.5 m depth using a horizontal Van Dorn water sampler and then transferred into 500 ml Nalgene bottles. A triple filter manifold with 0.45 µm filters was used to collect each of the following: suspended nitrogen (SuspN), suspended phosphorus (SuspP), and chlorophyll *a* (Chl *a*). The

filtrate was reserved for total dissolved nitrogen (TDN), total dissolved phosphorus (TDP) and dissolved organic carbon (DOC) analysis. The filters were desiccated at the ELA for 24 hrs, frozen, and shipped to the Freshwater Institute (FWI) Winnipeg, MB, Canada where analysis was completed. The filtrate analysis was completed at the ELA laboratory following procedures described in Stainton et al. (1977).

2.2.4.3. Water quality

Pre-treatment water quality parameters including temperature, dissolved oxygen (DO), specific conductivity, pH, and oxidation-reduction potential (redox) were monitored weekly beginning June 6, 2012 using a Quarda Stat HydroLab multi-probe.

Light attenuation profiles were collected bi-weekly from April 17 – August 29, 2012 as part of the long-term ELA Lake Sampling Program. Records were provided by Ken Sandilands, ELA Lake Sampling Program Manager.

2.2.4.4. POEA in the water column

A total of ten 250 ml water samples were collected in amber glass bottles directly below the surface of the water (~0.25 m depth); one from each open-bottom mesocosm and one from ~5 m outside the enclosures. A volume of 100 ml of each sample was filtered through a 1.2 µm glass microfibre filter. The filtrate was preserved at a ratio of 1:1 with methanol (100 ml) and stored at 4°C prior to analysis in amber glass.

2.2.4.5. POEA bound to suspended particulates

The 1.2 µm glass microfibre filters retained from each water sample were placed in labeled Whirl-Pak bags and stored at -20°C prior to extraction and analysis of POEA bound to

particulates.

2.2.4.6. POEA in sediments

A total of seven pre-treatment sediment cores measuring 3.0 cm high and 3.8 cm diameter (total volume: 58.5 cm^3) were taken from the boat using a custom designed coring apparatus (V. Palace, pers. comm.); one from each open bottom mesocosm and one from outside each enclosure. The cores were stored at -20°C prior to extraction and analysis of POEA concentration.

2.2.4.7. Zooplankton

The pre-treatment zooplankton community was sampled by taking duplicate 25 L Schindler-Patalas trap (total 50L) with a 53 µm filtering net from each mesocosm on June 11, 2012, pre-treatment. The top of the trap was lowered to just below the surface and thus, sampled the water column from 0 to 0.75 m in depth. In the field, samples from the Schindler-Patalas trap were transferred to 200 ml Nalgene bottles. In the lab, samples were further filtered through a 38 µm sieve and transferred to 40 ml glass vials with 2 ml of sugar formalin for preservation, identification, and quantification.

2.2.4.8. Fish

Free-swimming Fathead minnows were sampled from the open-lake to represent the pre-exposure period. Minnow traps were set overnight in L114 on July 23, one day prior to dosing the enclosures with POEA. A sub-sample of 10 males and 10 females (n = 20) were collected, weighed, and measured.

2.2.5. POEA treatment

Dose concentrations were prepared in the lab based on the mesocosms' estimated volume of 3140 L one day prior to treatment. A 1 $g.L^{-1}$ stock solution was prepared by adding 2815 µl of the 710 $g.L^{-1}$ POEA obtained from Monsanto (1 Research Rd, Winnipeg, MB R3T 6E3) into a 2 L volumetric flask. The solution was brought up to volume with distilled water and thoroughly mixed. When preparing treatments for the mesocosms, 31.4 ml of 1 $g.L^{-1}$ POEA stock solution was measured for the low dose mesocosms and 314 ml of the POEA stock solution was measured for the high dose mesocosms.

Each mesocosm treated with POEA received a one-time dose added in liquid from directly over the surface of the corral beginning at 8:53 a.m. CST on July 24, 2012. Dosing took approximately 1 hour. The low treatment mesocosms were dosed first, followed by the high treatment mesocosms. The mesocosms were dosed at the surface and mixed manually. Environmental conditions during dosing of the mesocosms were characterized as 50% cloud cover with low wind speeds.

2.2.6. Post Treatment Sampling and Analysis Endpoints

2.2.6.1. Nutrients

Water samples for post-treatment nutrient analysis were collected once a week for the first three weeks following treatments and biweekly thereafter until the completion of the experiment (October 8, 2012). Samples were analyzed for all nutrients described in Section 2.2.4.2.

2.2.6.2. Water quality

Post-treatment water quality parameters were monitored as described in Section 2.2.4.3 in parallel to water sub-sampling for POEA analysis.

2.2.6.3. POEA in the water column

Water samples were obtained (following the same procedures as described for pre-treatment sampling) from each enclosure at 1, 2, 4, 8, 16, 32, 48, and 72 hrs after POEA additions. After this time period, water sampling continued every second day until day 20 (August 16) of the experiment. Following the twentieth day, water sampling continued at a weekly interval until October 8, 2012. Analysis of POEA in the water samples were completed using liquid chromatography with tandem mass spectrometric detection methods (LC/MS/MS) (Ross and Liao, 2015). Samples taken at 1, 2, 4, 8, 16, 32, 48, and 72 hrs, as well as, days 5, 7, 9, 23, and 77 were analyzed at the Laboratory of Expertise for Aquatic Chemical Analysis (LEACA), a Department of Fisheries and Oceans (DFO) facility in Sydney, BC.

2.2.6.4. POEA bound to particulates

Extraction of POEA bound to particulates on the filters retained following filtration of the water samples, was completed at the FWI. The filters were placed in 20 ml glass scintillation vials and soaked in methanol for 24 hrs. Extracts were homogenized (manually shaken for 1 min) and combined with 1 ml HPLC grade water at a ratio of 1:1 water : MeOH. LC/MS/MS analysis for POEA bound to the particulates was completed at the University of Guelph Laboratory Services Division, Ontario, based on methods described by Ross and Liao (2015).

2.2.6.5. POEA in sediments

Sediment cores were taken following the same procedures as described for pre-treatment sampling from each of the open-bottom mesocosms (Section 2.2.4.6). The initial core was taken at 72 hrs post treatment, then weekly for 2 weeks following additions, and then biweekly until the completion of the experiment on October 8, 2012.

POEA extractions from the sediments were performed at the FWI using an accelerated solvent extraction (ASE) method (instrument: Dionex 200 ASE), a technique for the extraction of organic compounds from solid and semisolid sample media using common solvents at elevated temperatures and pressures. Samples were prepared for extraction following the protocol described by Ross and Liao (2015). Due to the high moisture content (> 90%) of the L114 sediment, a 5 ml sample was used instead of the 3 g sample suggested. The Dionex 200 ASE instrument settings for extraction were: preheat (1 min), heat (6 min), static (10 min), and flush (75%), purge 120sec, temperature (120 °C), pressure (1200 psig), and the number of cycles (2). The extraction solvents consisted of 150 ml of 100% MeOH, 150 ml KH2PO4 (0.1 M aq), 150 ml HPLC water, and 1500 ml MeOH. After heating, the extract was flushed from the sample cell into a standard collection vial. Final volumes of extract in the collection vials were recorded.

LC/MS/MS analysis of POEA sediment extracts for July 27, August 2, and August 9 were performed at the LEACA. Sediment extracts for October 4, 2012 were analyzed at the University of Guelph Laboratory Services Division, Ontario.

2.2.6.6. Zooplankton

Post-treatment zooplankton community was sampled following the same methods as

described in Section 2.2.4.7. Zooplankton was collected once a week for the first three weeks following treatments, and biweekly thereafter until the completion of the experiment (October 8, 2012).

2.2.6.7. Fish

Fathead minnows captured from L114 were deployed in each of the mesocosms (n = 10) on July 23, 2012, one day prior to POEA dosing. They were fed fish flakes for the duration of the experiment at a ration of 2% bodyweight, four times per week. This ration has been used in fish held in 2 m diameter limnocorrals and documented growth similar to non-captive fish in the same lake during previous experiments (V. Palace, pers. comm.). Estimated survival rate was > 80%.

Fathead minnows from each mesocosm were sampled after 1, 2, and 4 weeks of independent exposure periods to POEA. Fish added to the enclosures for subsequent 2 and 4 week exposures were captured in L114 with minnow traps. Prior to adding the fish to the enclosures, fish were marked by clipping fins to ensure the identity of the exposure duration. The dates for the fish exposure periods were; July 24 - 31 for the 1 week exposure; July 31 - August 14 for the 2 week exposure, and August 14 - September 11 for the 4 week exposure. This approach was used, as opposed to a single fish addition with subsequent subsampling through time, to mimic the effects of aged POEA on fish. The alternative of adding fish only at the beginning of the experiment was not used to decrease the possibility of a density-dependent response in fish growth. In other words, fish growth could have been affected by limited resources for the first period (when densities were greatest), and a relative increase in resource availability later on when fish densities were lowered by subsampling. Fish were recaptured from

the mesocosms using baited minnow traps (Appendix D).

2.2.7. Data Analysis

2.2.7.1. Nutrient analysis

Statistical comparisons of nutrient data between mesocosms was not conducted given the constraints of only having two mesocosms per treatment group. However, trends in the data relative to the controls are presented.

Trophic status was evaluated using the phosphorus and chlorophyll α criteria as described by the Organization for Economic Co-Operation and Development (OECD, 1982). Total nitrogen corresponds to the Nurnberg criteria (Galvez-Cloutier & Sanchez, 2007).

The sum of Susp P and TDP is described as total phosphorus (TP). The sum of Susp N and TDN is described as total nitrogen (TN). Total organic carbon (TOC) was calculated by combining dissolved and particulate organic carbon (DOC and Susp C) concentrations.

The TSS concentration in samples calculated using the following equation (M. Pateron, pers. comm):

$$TSS\ (mg.L^{-1}) = -0.95 + (0.003 * Susp\ C\ (\mu g.L^{-1})$$

The equation was derived from simultaneous measures of Susp C and TSS taken from all research lakes at ELA from 1993 – 1996 ($R=0.76$; $n=400$; $p<.0001$) (M. Paterson, pers. comm.).

2.2.7.2. Water quality analysis

Statistical comparisons of water quality data between mesocosms was not conducted

given the constraints of only having two mesocosms per treatment group. However, trends in the data relative to the controls are presented.

2.2.7.3. POEA in the water column

The DT50s were calculated based on one-time treatment concentrations (0 $\mu g.L^{-1}$, 10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$), and the loss of POEA at all measurable treatment concentrations was described by linear regression analysis of concentration versus time. Since the concentration data was not homoscedastic, it was transformed using a natural log scale. Dissipation times were determined for each mesocosm as well as mean POEA concentration over time in both high and low treatments at the first sample point at which POEA was below the limit of quantification (< LOQ) for each mesocosm (mean low = 16 hrs, mean high = 72 hrs).

A generalized estimating equation (GEE) was constructed for POEA concentrations in water over time (0.90 confidence interval). This model is semi-parametric in that it estimates the parameters parametrically while the variances are estimated non-parametrically. The parameter estimates produced by this method evaluate the effects of time and POEA treatment level and the interaction between the two variables on the data. The interaction term is incorporated to determine if the effect of time depends on the contaminate level. An autoregressive correlation structure was used to account for the greater correlation in data points that are closer in time than data points which are farther away (Koper & Manseau, 2010). A GEE was first run with only the response variable, POEA concentration in water, and the two independent variables, time and POEA treatment level. Univariate analysis on the residuals determined that these data were not distributed normally. The POEA concentration data (y - axis) was log transformed to meet this assumption. The scale parameter for GEE estimation was computed as the square root of the

normalized Pearson's chi-square. Results were considered significant if p value < 0.05. All analyses were performed with SAS 9.4.

2.2.7.4. POEA bound to suspended particulates

Concentrations of POEA bound to particulates are presented weight/weight (w/w) based on the TSS concentration in samples on July 25, 2012.

$$POEA(mg.g^{-1}) = \frac{\{[(POEA(mg.L^{-1}) x\ DF) - Blank] x\ Final\ Volume\ (L)\}}{\text{TSS (g)}}$$

Where, POEA ($mg.L^{-1}$) = raw value ($ng.ml^{-1}$)/1000, DF = 2, Final Volume = 0.02 L, TSS = ($mg.100\ L^{-1}$)/1000.

The data was transformed and the DT50 of POEA bound to suspended particulates was calculated as described in Section 2.2.7.3.

2.2.7.5. POEA in the sediment

Sediment data received from the LEACA and University of Guelph Laboratory Services Division, was corrected using the following equation:

$$POEA(ng.g^{-1}) = \frac{\{[(POEA(ng.ml^{-1}) x\ DF) - Blank] x\ Final\ Volume\ (ml)\}}{\text{Mass of Sample (g)}}$$

Where, POEA ($ng.ml^{-1}$) = raw value, DF = 1 (no dilution), Mass of Sample (g) = 5 ml - (% moisture * 5 ml).

The DT50s were calculated on the mean POEA concentration in aquatic sediments over time in both high and low treatments, as described in Section 2.2.7.3.

2.2.7.6. Fish

Fish condition was calculated based on fork length and weight measurements using Fulton's Condition Factor (FCF) (K), which measures the health of a fish assuming that the standard weight (W) of a fish is proportional to the cube of its length (L^3) (Fulton, 1904). A scaling factor is usually applied to bring the factor close to 1. In this case a scaling factor of 100 was used (Froese, 2006). Recovered mortalities were automatically assigned a condition factor of zero. The FCF can be calculated using the following equation (Fulton, 1904):

$$K = 100 * W/L^3$$

Statistical comparisons of FCF data between mesocosms was not conducted given the constraints of only having two mesocosms per treatment group. However, trends in the data relative to the controls are presented.

2.3. Results

POEA investigated in all ecosystem compartments; water column, suspended particulates, and aquatic sediments, exhibited a decreasing trend through time. The greatest initial concentration of POEA was bound to suspended particulates, but no longer detectable after 72 hrs. POEA decreased rapidly in the water column and was below the limit of quantification in all mesocosms by the ninth day following treatment. POEA was still detectable in the sediment of enclosures treated with 100 $\mu g.L^{-1}$ on the final day (day 77) of sample collection.

2.3.1. Weather monitoring

All weather data was provided by Ken Beaty, ELA Hydrologist. Detailed metrological data for the duration of the experiment July 24 – October 8, 2012 are presented in Appendix A.

2.3.2. Nutrients

Nutrients monitored included: Susp P, TDP, Susp N, TDN, Susp C, DOC, TOC, and Chl α (Appendix B). No POEA treatment effects were observed on nutrients.

Total phosphorus in L114 decreased from June 11 - October 2, 2012 (25 - 15 $\mu g.L^{-1}$) (Appendix B: Table B.1). No trends are observed between the controls and the surrounding lake, nor were any trends observed between low or high treatments. Closed-bottom designs maintained elevated levels of TP (29 - 53 $\mu g.L^{-1}$) at the end of the study, whereas by October 2, all open-bottom designs returned to levels consistent with the surrounding lake (15 - 20 $\mu g.L^{-1}$).

No trends were observed in TN between the controls and the surrounding lake, nor were any trends observed between low or high treatments (Appendix B: Table B.2). Closed-bottom designs maintained elevated levels of total nitrogen (902 - 973 $\mu g.L^{-1}$) at the end of the study, whereas by October 2, all open-bottom designs returned to levels consistent with the surrounding lake (717 - 799 $\mu g.L^{-1}$) (except Low A, 879 $\mu g.L^{-1}$). Particularly high levels of TN was recorded in pre-treatment samples of Control A (1285 $\mu g.L^{-1}$). Control B, Low B and High A also had elevated TN levels on July 25 (1722, 2399, and 1789, respectively). Elevated TN concentrations relative the other mesocosms and L114 persisted until July 31 in Control B and until August 7 in Low B.

Variability in TOC concentrations were observed in all mesocosms and the surrounding lake

(Appendix B: Table B.3). However, recorded TOC followed the same trend in each mesocosm. Total organic carbon increased from June 11 – September 18, and dropped off by October 2, 2012. In L114, TOC peaked on September 4 and remained elevated, relative to the mesocosms, until the end of the study. Large spikes in DOC where observed in Control C on July 25 (88800 $\mu g.L^{-1}$) and in L114 on August 7 (85400 $\mu g.L^{-1}$), which were likely due to an analytical error. A notable measure of Susp C was observed in High A on September 18, 2012 (13620 $\mu g.L^{-1}$). Total suspended solids calculate from Susp C concentrations on July 25, 2015 ranged from 2.2 – 11.2 $mg.L^{-1}$ in mesocosms as compared to 8.8 $mg.L^{-1}$.

Although variability in Chl α concentrations were observed, the general trend in all treatments was a normal distribution with slight positive or negative skews (Appendix B: Table B.4). No trends in Chl α concentration were attributed to POEA treatments.

Light attenuation coefficient (k) in L114 on May 23 was 0.8 m^{-1} and 1.0 m^{-1} on July 18, 2012.

2.3.3. Water Quality

No POEA treatment effects were observed on water quality parameters (Appendix C). Measured values for temperature (Table C.1), specific conductivity (Table C.2), and redox potential (Table C.5) were similar to those observed in L114 over the duration of the monitoring period (June 6 - October 11, 2012).

Variability in DO concentration is observed prior to treatments from June 6 – July 18, 2012 in the open-bottom mesocosms (Appendix C: Table C.3). The closed-bottom mesocosms maintained DO similar to the surrounding lake over the entire period monitored. Lowest DO, post POEA treatment, was observed in Control A on July 25 (4.8 $mg.L^{-1}$). On August 15, High B

also had relatively low DO (4.7 $mg.L^{-1}$). In comparison, L114 lowest recorded level post POEA treatment was 6.7 $mg.L^{-1}$ on August 21. In October, DO increased in all of the enclosures and L114 as temperatures decreased. Final DO concentrations ranged from 10.1 – 10.7 $mg.L^{-1}$ on October 11.

The pH measurements recorded in the mesocosms and surrounding lake ranged from 4.7 - 8.1 from June 6, 2012 to October 11, 2012 (Appendix C: Table C.4). Trends observed in enclosures were similar to those observed in L114 from June 6 - October 11, 2012. A slight increase in treated enclosures (except High B) was observed on July 25, the day following POEA treatment. The pH, post POEA treatment (July 24 - October 11), peaked in all enclosures on September 11 (mean 7.6 ± 0.3), then dropped and plateaued from September 18 - October 11 (mean 5.5 ± 0.4). Control A maintained the lowest pH, post treatment, from July 25 - September 11 (mean 5.8 ± 0.8), while High A was the highest (mean 7.1 ± 0.9). In comparison, from July 24 - September 11, the mean pH in L114 was 6.4 ± 0.7.

2.3.4. POEA in the water column

Results show relative variation in POEA concentrations through time in each enclosure (Table 2.1, Figure 2.3). In open-bottom corrals, which received a high treatment (POEA concentration of 100 $\mu g.L^{-1}$), a second peak in concentration occurred at 120 hrs (79 $\mu g\ L^{-1}$) and 168 hrs (23 $\mu g\ L^{-1}$), in High B and A, respectively.

Samples were collected on the twenty-third and seventy-seventh day of exposure to ensure that remobilization of the POEA did not persist over the long-term. These samples confirmed that POEA in the water column was below detection levels. In the closed-bottom, high treatment enclosure (High C), a second peak in POEA concentration was also observed at 168 hrs, but the

increase appears to be less substantial than in open-bottom, high treatment enclosures.

The DT50 of POEA varied between treatments, as well as individual mesocosms (Table 2.2 & Figure 2.4). In open-bottom enclosures, which received high treatments, the DT50 of POEA was 11.3 hrs and 15.2 hrs in High B and A, respectively (mean DT50 = 14.8 hrs). Similarly, in the closed-bottom enclosure, which received the high treatment dose (High C), POEA had a DT50 of 16.2 hrs. In the open-bottom enclosures that received low treatments, the DT50 of POEA was 2.6 hrs and 3.3 hrs in Low A and B, respectively (mean DT50 = 3.2 hrs). In the closed-bottom design, Low C, POEA exhibited a DT50 of 1 hr. Total suspended solids did not appear to affect the dissipation time of POEA (Figure 2.5).

Due to lack of replicates, open and closed bottom mesocosm designs were grouped for the generalized estimating equations based on dose treatment without regard to mesocosm design. The distribution of the log transformed data still exhibits a slight positive skew, but is adequate for GEE analysis.

The model-based standard error estimates of the GEE for the change of POEA overtime showed a significant difference ($p = <0.0001$) between the high treatment and the control (Table 2.3). Significance ($p = 0.0043$) was also observed in the time and treatment interaction parameter (hr*treatment) between the high treatment and control.

2.3.5. POEA bound to suspended particulates

Dissipation of POEA in all treatments exhibited an exponential decrease over time (Table 2.4 & Figure 2.6, Figure 2.7). Mean initial detectable level in open-bottom high treatment corrals was 14.9 $mg.g^{-1}$ POEA (DT50 = 9.1 hrs), and 19.6 $mg.g^{-1}$ POEA (DT50 = 14.7 hrs) in the closed-

bottom design. In open-bottom low treatment corrals, the mean initial detectable level was 1.5 $mg.g^{-1}$ POEA (DT50 = 5.9 hrs), and 2.23 $mg.g^{-1}$ POEA (DT50 = 7.0 hrs) in the closed-bottom design. Dissipation times of POEA bound to particulates was slightly longer in the closed-bottom design than in their open-bottom counterparts. POEA bound to particulates was no longer detectable by 72 hrs with the exception of open-bottom High B and closed-bottom High C mesocosms, in which levels were very low. POEA concentrations observed in controls and on the seventy-seventh day of the experiment are likely due to an analytical error or sample contamination during analysis.

2.3.6. POEA in the sediment

POEA values from 155.3 - 369.3 $ng.g^{-1}$ were present in Control B and the surrounding lake in the first week (Table 2.5 & Figure 2.8, Figure 2.9). High A had the greatest initial concentration of POEA in the sediment (4731.5 $ng.g^{-1}$) after 3 days. In contrast, initial POEA concentrations in the sediment of Low A and B, and High B, where similar, ranging between 380.8 and 553.2 $ng.g^{-1}$. In High B, the POEA concentration (5530.2 $ng.g^{-1}$) did not approach values initially observed in High A until the fourteenth day following treatment. The final samples analyzed from October 8, after 77 days from initial dosing, still had detectable levels of POEA in both high treatment mesocosms, 389.9 and 156.55 $ng.g^{-1}$ in A and B, respectively.

The DT50 of POEA in the sediments was only calculated in High A due to the lag observed in High B and lack of subsequent samples (DT50 = 42.5 days). The DT50 of mean POEA in the sediment of Low treatment corrals was 8.8 days.

2.3.7. POEA treatment effects on zooplankton

Microscopic analysis at magnification of 25X – 50X revealed substantial decreases (~95% less) in zooplankton densities in all of the samples obtained from all the enclosures (inclusive of controls) throughout the duration of the study as compared to samples taken from the surrounding lake. Abundance and species composition data are not reported given the vast reduction in these metrics in all corrals.

2.3.8. POEA treatment effects on fish

Archived measurements of pre-exposure fish from L114 were as follows: male total fork length (mean ± SD) was 5.64 ± 0.13 cm, weight was 2.48 ± 0.36 g, and FCF was 1.38 ± 0.16. For females, total fork length was 5.37 ± 0.48 cm, weight was 2.15 ± 0.52, and FCF was 1.37 ± 0.15 (Appendix D). A Student's t-test confirmed that there was no significant difference between the mean condition of the pre-exposure sub-sample male and female fathead minnows ($p = 0.921$)

During the 1 week exposure (n = 90) (July 24 - 31), there were a total of 8 mortalities (7%) (Table 2.6). During the 2 week exposure (July 31 - August 14) there were a total of 15 mortalities (17%), with 7 observed from Control C. During the 4 week exposure (August 14 - September 11) no mortalities were observed.

Recapture success (recovered mortalities inclusive) decreased as exposure duration increased. Following the 1 week exposure, 94% (85/90) of the Fathead minnow population was recovered. After the 2 week exposure, 88% (79/90) were recaptured; and after 4 weeks, 83% (75/90).

The mean total fork length of fish recaptured after 1 week POEA exposure was 5.8 ± 0.4 cm (range: 4.7 - 7.2 cm), weight was 2.6 ± 0.7 (1.1 - 5.5 g), Fulton's condition factor was 1.18 ± 0.4.

The mean total fork length of fish recaptured after 2 week POEA exposure was 5.7 ± 0.5 cm (4.6 - 6.9 cm), weight was 2.4 ± 0.6 g (1.3 - 3.5 g), Fulton's condition factor was 1.04 ± 0.5. The mean total fork length of fish recaptured after 4 week POEA exposure was 6.0 ± 0.3 cm (5.2 – 7.4 cm), weight was 2.5 ± 0.5 g (1.5 - 4.6 g), Fulton's condition factor was 1.14 ± 0.1.

2.4. Discussion

2.4.1. Nutrients and water quality

The mesocosms were deployed May 16, 2012 and were not dosed with POEA until July 24, 2012. This two month period, necessary for adequate periphyton colonization, also provided time for other ecological parameters to differentiate. It is possible that this time lag between the deployment of enclosures and dosing of treatments caused deviations to occur in several parameters. Variations in nutrients, pH and DO were observed as well as in biota. The presence or lack of macrophytes and periphyton may also have contributed to the variability in the fate of POEA. Environmental parameters measured in the enclosures are reviewed.

In this experiment, the presence or absence of underlying sediment and the amount of TOC in each mesocosm did not appear to influence the fate of POEA, as previously suggested (Giesy et al. 2000, Wang et al. 2005). Total organic carbon was predicted to be a factor that could increase the degradation rate of POEA in water. The dissipation time of POEA was not found to be inversely proportional to TOC concentrations in the water column of the mesocosms. Light attenuation is directly correlated with DOC and TSS. Lake 114 itself has a very high TSS concentration (light attenuation coefficient = 0.8 m^{-1} on May 23, 1 week after mesocosm deployment). A multiple site study that considers various lake characteristics (such as rocky

bottom and low TSS) would be necessary to effectively assess the affinity of POEA to suspended particulates or high organic content in natural systems.

Elevated levels of nitrogen were observed in Control B, Low B and High A on July 25, 2012. This coincided with the lowest recorded redox values observed in the same corrals. A lower redox value, although not negative, would indicate a more reducing environment than recorded at any other sampling time. It is unclear why these particular corrals experienced this flux in nitrogen. Each one received a different treatment, so therefore it cannot be attributed to the secondary amine intermediate from the bacterial degradation of POEA as described by Van Ginkel et al. (1993). Furthermore, POEA is not suspected to have affected nitrogen levels as these fluxes were not observed in the other treated corrals. The location of the mesocosms may have contributed to the nitrogen patterns observed. Control B, Low B and High A were closer to the outflow compared to the other enclosures. It is possible that sediment characteristics were slightly different near the outflow.

As expected, another correlation observed was between suspended phosphorus and Chl α. Elevated levels of both occurred on June 11, 2012, pre-POEA exposure, in Control A and B, and Low A and B. By July 25, Chl α concentrations were reduced while suspended phosphorus concentrations remained elevated. It is possible that some phytoplankton died but remained suspended in the water column. The reduction in Chl α is not attributed to POEA treatment because the highest levels of Chl α remained in the high treatment corrals after dosing.

For the most part, all water quality parameters followed the same trends as samples collected outside of the mesocosms, from L114. Although variations were not found to be correlated to POEA dose levels, some correlations between parameters, such as temperature and DO, were

observed as expected.

2.4.2. POEA in the water column

Variations observed in the DT50 between enclosures are likely attributed to the environment itself. Previous studies have noted that interaction with organic matter, sediments, and suspended particulates may affect the DT50 of POEA in the water column (NRA 1996, Giesy et al. 2000, Wang et al. 2005). The DT50 of POEA determined in high treatment mesocosms (mean DT50 = 14.8 hrs) was similar to that reported in the microcosm study by Wang et al. (2005) (DT50 = 13 – 18 hrs). Furthermore, POEA dose concentrations were calculated based on an assumed volume of 3140 L. It is likely that the volume of water in each mesocosm was not exactly equal to the assumed volume. Variations in water volume between mesocosms would have affected POEA concentrations at time.

The possible re-suspension of POEA was modeled in the high treatment mesocosms because it was more distinct than in the low treatment enclosures. This phenomenon may be attributed to environmental conditions or may simply be an analytical error. Evidence for the former is supported through the metrological data from July 24 – 31, 2012. The high volume of rainfall recorded on July 25 (25.5 mm), the second day of POEA exposure, may have diluted POEA concentration in surface water samples. Furthermore, on July 26, winds reaching 18 $km.hr^{-1}$ from an ESE direction (the maximum fetch of L114) may have stirred up settled POEA and effectively agitated particulates to release bound POEA. However, concentrations of POEA in the sediment and bound to particulates do not show a counter trend to support this hypothesis. Thus, it must be concluded that spikes in POEA concentration in the high treatment corrals on the fifth and seventh day of exposure are due to analytical error.

The GEE deals with the correlation caused by collecting numerous samples from each mesocosm by adjusting the standard error to compensate for the lack of independence among samples. It is important to note that transformations (ie. log) can reduce or remove the effect of interactions, which was observed in the POEA model.

2.4.3. POEA bound to particulates

There appeared to be no notable differences between concentrations of POEA bound to particulates in open or closed bottom designs. Water pumped from the surrounding lake into the closed-bottom designs still would have had a high level of particulates for POEA to bind to, regardless of the presence of underlying sediments. The prediction that POEA may have a high affinity for suspended particulates, potentially leading to the sedimentation of POEA from the water column to the bottom sediments, cannot accurately be assessed based on this experimental design. It is also important to note that the analysis of POEA in the water column and bound to particulates was conducted at two different labs. It is possible that slight modifications to methods may have been made to accommodate the particular abilities and amenities of each lab.

2.4.4. POEA in the sediment

Variability between enclosures may be due to how POEA partitioned. The cores were taken at different locations within the corral each time. If the composition of underlying sediments was not homogenous throughout the mesocosm, it may be that POEA selectively bound to sites with a greater organic content as suggested by Wang et al. (2005).

There is evidence of diffusion of POEA through sediments given its presence in the surrounding lake and Control B. Samples were taken from different locations in the surrounding lake each

time; however, funding limitations allowed for only a select number of samples to be analyzed. It is difficult to conclude if diffusion is taking place or if contamination occurred during analysis with limited sample size. Again, sediment samples were conducted in part by two different labs. As stated in Section 2.4.3, this may pose analytical inconsistency in the resultant data from each lab.

Mass balance budgets were not conducted to determine mass flow of POEA in the mesocosm systems. There were an insufficient number of samples analyzed in each ecological compartment to justify the application of this evaluation. The concentration of POEA that exceeded dose levels bound to suspended particulates in early sample points (1 and 4 hrs) may have been the result of inadequate mixing within the mesocosms, or inaccurate estimation of mesocosm volume. Dose solutions were made up to equal 10 $\mu g.L^{-1}$ or 100 $\mu g.L^{-1}$ POEA in estimated 3140 L volume of the enclosures, but an actual volume measurement was not taken (Section 2.2.5). Surface applications of POEA were closely followed by sample collection at the surface leading to highly concentrated levels of POEA in the water and bound to particulates in initial samples.

2.4.5. POEA treatment effects on zooplankton and fish

Survival and mortality of fish in the mesocosms were likely not related to POEA exposure as fish mortality was equally observed in the control enclosures. In an exposure experiment, freshwater fish exposed to MON 0818 (comprised of 75% POEA) for a 96 hr period showed LC50 values between 1 and 13 $mg.L^{-1}$ (Folmar et al., 1979). During our mesocosm experiment, exposure concentrations of POEA were at least an order of magnitude lower than LC50 values reported by Folmar et al. (1979). It is therefore unlikely that POEA concentrations in the enclosures were responsible for the observed fish mortality.

Fish condition, following the 2 and 4 week exposures, was not expected to be directly affected by POEA due to the short DT50 of POEA in the water. Interestingly, the results show a decrease in the condition of fish in Low A and C (10 µg.L^{-1} POEA) sampled from the 2 week POEA exposure, as compared to the lake sub-sample. Vandenberg et al. (2012), postulated that endocrine-disrupting compounds could have effects at low doses that are not predicted by effects at higher doses, this hypothesis has not yet been readily studied in relation to surfactants. This could indicate an effect from aged POEA; however, POEA was no longer detectable in the water column of all mesocosms after the ninth day of exposures; and therefore, reduced condition is unlikely attributed to POEA.

Moreover, other factors such as water quality parameters DO, pH, nitrogen compounds, and others varied between the enclosures and could also have contributed to fish condition. In a study conducted by Wan et al., (1989), five Salmonid species where exposed to four pesticide compounds in water from five different sources, which ranged from soft city water to hard lake water. The results indicated that the toxicity of POEA and Roundup® increased as conductivity, hardness, and pH increased, whereas the toxicity of glyphosate decreased in all species. In the present study, DO, pH, nitrogen compounds, and others varied between the enclosures. However, these parameters did not differ substantially during the 2 week exposure in Low A and C from the mean of all enclosures and the surrounding lake. Specific conductivity and pH measured in the enclosures did not reach levels noted by Wan et al. (1989) to increase the toxicity of POEA. Therefore, it is unlikely that these parameters were responsible for the decrease in FCF or the observed mortalities.

Indirect effects are possible if POEA affected fish food supply even with supplementary fish food added to the enclosures. A change in food supply could have caused decreased weight in

fish. In fact, zooplankton densities were scarce in mesocosms as compared to the surrounding lake. However, observations of zooplankton densities did not show differences that were correlated to POEA concentrations, as all corrals had substantial decreases. The observed decline in zooplankton density may have been due to predation by *Chaoborus* larvae on the confined community (Neill, 1981; Vanni, 1988).

Short acclimation time in the mesocosms and handling stress on fish prior to exposure may also be accountable for observed mortalities (Horton, 1956; Barton & Iwama, 1991). For instance, during the 2 week exposure, eight mortalities were observed on the first day of the exposure. Mesocosm studies in general have both advantages and disadvantages. The idea of a mesocosm study is to extrapolate to whole lake settings. However, there are so many variables which cannot be controlled in order to mimic the lake entirely, which was observed in the variability of nutrients, periphyton colonization, and the decline in zooplankton communities. It's possible that these differences from the surrounding lake can all be attributed to the duration the mesocosms were left in place (Lund 1972; Smyly 1976; Levine & Schindler 1992; Schindler 1998). Combined, these factors may have contributed to fish condition and, in the present experimental design, cannot be associated to POEA toxicity.

2.5. Conclusion

The presence of POEA was determined to be short-lived in the water column and more extended in the sediments. Based on the results of this study, TSS had no sizable influence of the fate of POEA. The fate of POEA in sediments and the toxicity to benthic organisms are still poorly understood. The fact that POEA is still present in the sediments more than two months after a one-time dose suggested that there may be a significant threat to benthic organisms as proposed

by Giesy et al., (2000). Furthermore, in a true agricultural setting, glyphosate-based herbicides may be applied multiple times throughout the season: (1) Pre-seed burn-off of weeds in the spring, (2) up to two applications for pre-harvest weed control, (3) harvest desiccation, and (4) a post-harvest burn-off of weeds in the fall. Multiple doses of POEA to aquatic environments should be investigated. As should the degradation of POEA in aquatic sediments over winter, following multiple doses, to assess for season-to-season accumulation.

In the literature, very limited data was available for POEA toxicities; whereas the range and density of species exposed to glyphosate and its subsequent formulation was much more robust. It is unlikely that POEA would enter the environment unaccompanied by the active ingredient, glyphosate. This suggests that environmental monitoring data for POEA is needed.

Table 2.1. POEA concentrations ($\mu g.L^{-1}$) in the water column of mesocosms positioned in L114 at the ELA from July 24 - October 10, 2012. Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA. LOQ: Limit of quantification.

DAY	HR	Control A	Control B	**Mean**	Low A	Low B	**Mean**	High A	High B	**Mean**	Control C	Low C	High C
1	1	<LOQ	<LOQ	**<LOQ**	6.1	5.92	**6.0**	123.2	47.16	**85.2**	<LOQ	1.3	52.0
1	2	9.6	3.4	**6.5**	3.6	5.73	**4.7**	59.6	43	**51.3**	3.7	1.5	42.0
1	4	<LOQ	16.56	**8.3**	1.4	8.15	**4.8**	74.4	54.89	**64.6**	<LOQ	1.2	45.4
1	8	2.8	<LOQ	**1.4**	1.6	4.41	**3.0**	34.06	34.24	**34.2**	<LOQ	<LOQ	18.3
1	16	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	45.89	21.39	**33.6**	<LOQ	<LOQ	39.7
2	32	1.9	<LOQ	**1.0**	<LOQ	2.4	**1.2**	29.4	24	**26.7**	<LOQ	<LOQ	36.0
2	48	2.2	<LOQ	**1.1**	<LOQ	<LOQ	**<LOQ**	13.8	<LOQ	**6.9**	<LOQ	<LOQ	14.2
3	72	<LOQ	<LOQ	**<LOQ**	<LOQ	0.98	**0.49**	4.08	<LOQ	**1.7**	<LOQ	<LOQ	4.8
5	120	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	79	**39.5**	<LOQ	<LOQ	5.2
7	168	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	23.2	23	**23.1**	<LOQ	<LOQ	10.8
9	216	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ
23	552	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ
77	1848	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ

Table 2.2. Physical observations made for each mesocosm pre-treatment on July 18, 2012. Periphyton colonization assigned a score of 0 – 5, where 0 was no visible growth on polyethylene strips suspended into each mesocosm and 5 was complete algal coverage of polyethylene strips. Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA. Calculated DT50 of POEA provided for each mesocosm.

	Periphyton Colonization	DT50 (hrs)	Observations
Control A	0		Slight green tinge, murky
Control B	3		Vegetation sprouting from sediment (< Low B)
Low A	1	2.6	Slightly murky
Low B	3	3.3	Clear, vegetation sprouting from sediment
High A	5	15.2	Very clear, overgrown with bladderwort
High B	2	11.3	Three lily pads, very murky
Control C	3		Murky, green tinge, removed a snapping turtle a week prior
Low C	1	1.0	Clear, lots of rotten pollen settled on the bottom
High C	3	16.2	Clear, lots of rotten pollen settled on the bottom

Table 2.3. Analysis of Generalized Estimating Equation parameter estimates for the change in mean POEA over time. ND means no data available.

Model-Based Standard Error Estimates							
Parameter		Estimate	Standard Error	90% Confidence Limits		Z	Pr > |Z|
Intercept		-0.8072	0.3533	-1.3883	-0.2262	-2.29	0.0223
Hr		-0.0001	0.0005	-0.0009	0.0007	-0.23	0.8157
Treatment	HIGH	2.9845	0.4996	2.1628	3.8063	5.97	<.0001
Treatment	LOW	0.3954	0.4996	-0.4263	1.2172	0.79	0.4287
Treatment	CONTROL	0.0000	0.0000	0.0000	0.0000	ND	ND
Hr*Treatment	HIGH	-0.0020	0.0007	-0.0032	-0.0009	-2.86	0.0043
Hr*Treatment	LOW	-0.0003	0.0007	-0.0015	0.0009	-0.41	0.6826
Hr*Treatment	CONTROL	0.0000	0.0000	0.0000	0.0000	ND	ND

Table 2.4. POEA concentration ($mg.g^{-1}$) bound to suspended particulates. Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA. LOQ: Limit of quantification.

DAY	HR	Control A	Control B	**Mean**	Low A	Low B	**Mean**	High A	High B	**Mean**	Control C	Low C	High C
1	1	<LOQ	<LOQ	**<LOQ**	1.39	1.66	**1.52**	8.70	21.03	**14.87**	<LOQ	2.23	19.61
1	4	0.12	0.20	**0.16**	0.86	2.00	**1.43**	5.80	16.37	**11.09**	<LOQ	1.32	21.60
1	16	0.02	0.02	**0.02**	0.13	0.33	**0.23**	2.75	10.67	**6.71**	<LOQ	0.47	19.41
3	72	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	0.11	**0.06**	<LOQ	<LOQ	0.55
5	120	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	<LOQ	0.02	**0.01**	0.57	<LOQ	0.03
77	1848	0.74	<LOQ	**0.37**	0.63	<LOQ	**0.32**	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	<LOQ

Table 2.5. POEA concentration ($ng.g^{-1}$) in sediments of each open-bottom mesocosms. Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA. LOQ: Limit of quantification. ND means no data available.

DAY	HR	Lake 114	Control A	Control B	**Mean**	Low A	Low B	**Mean**	High A	High B	**Mean**
3	72	275.3	<LOQ	155.3	**77.7**	380.8	553.2	**467.0**	4731.5	511.2	**2621.4**
7	168	ND	<LOQ	369.3	**184.7**	493.8	234.9	**364.4**	1667.6	2195.7	**1931.7**
14	336	ND	<LOQ	<LOQ	**<LOQ**	220.0	<LOQ	**110.0**	1204.9	5530.2	**3367.6**
71	1704	ND	<LOQ	<LOQ	**<LOQ**	<LOQ	<LOQ	**<LOQ**	389.90	156.55	**273.2**

Table 2.6. Fathead minnow mortalities and recapture success in each mesocosm during 1, 2, and 4 week exposures. Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA. Recaptured values are inclusive of mortalities.

	Mortalities			# Recaptured		
	1 week	2 week	4 week	1 week	2 week	4 weeks
Control A	1	2	0	8	8	9
Control B	0	0	0	9	7	9
Low A	2	0	0	10	9	9
Low B	0	2	0	10	8	4
High A	0	0	0	9	10	10
High B	1	1	0	9	9	9
Control C	3	7	0	10	10	9
Low C	0	3	0	10	10	7
High C	0	0	0	10	8	9
Sum	7	15	0	85	79	75
%	8%	17%	0%	94%	88%	83%

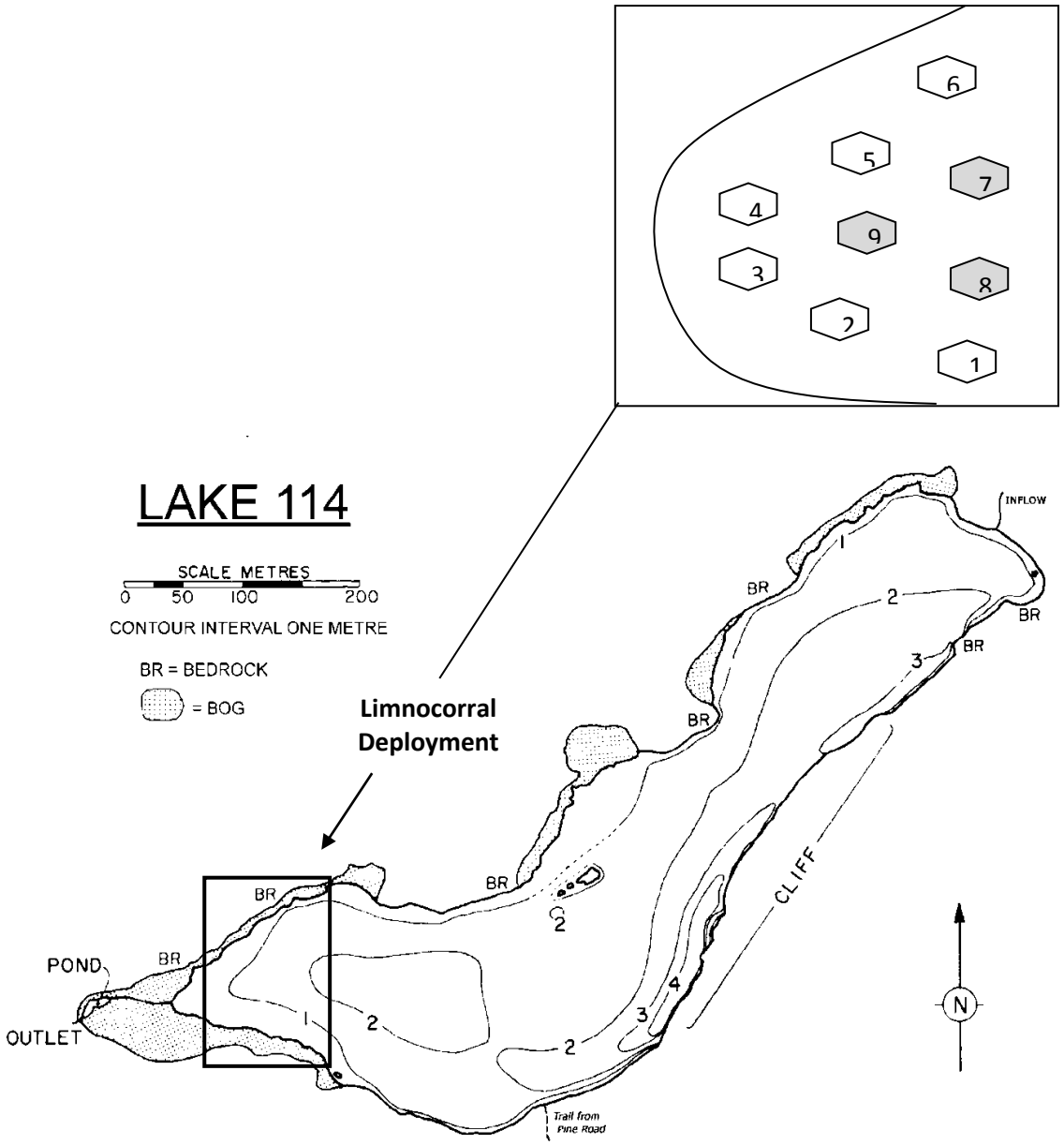

Figure 2.1. Bathymetric map of Lake 114 with inset of mesocosm distribution. Closed-bottom mesocosms are shaded. Corral numbers correspond to treatments as follows: 1 = Low A, 2 = Control A, 3 = High A, 4 = Low B, 5 = Control B, 6 = High B, 7 = Low C, 8 = High C, 9 = Control C.

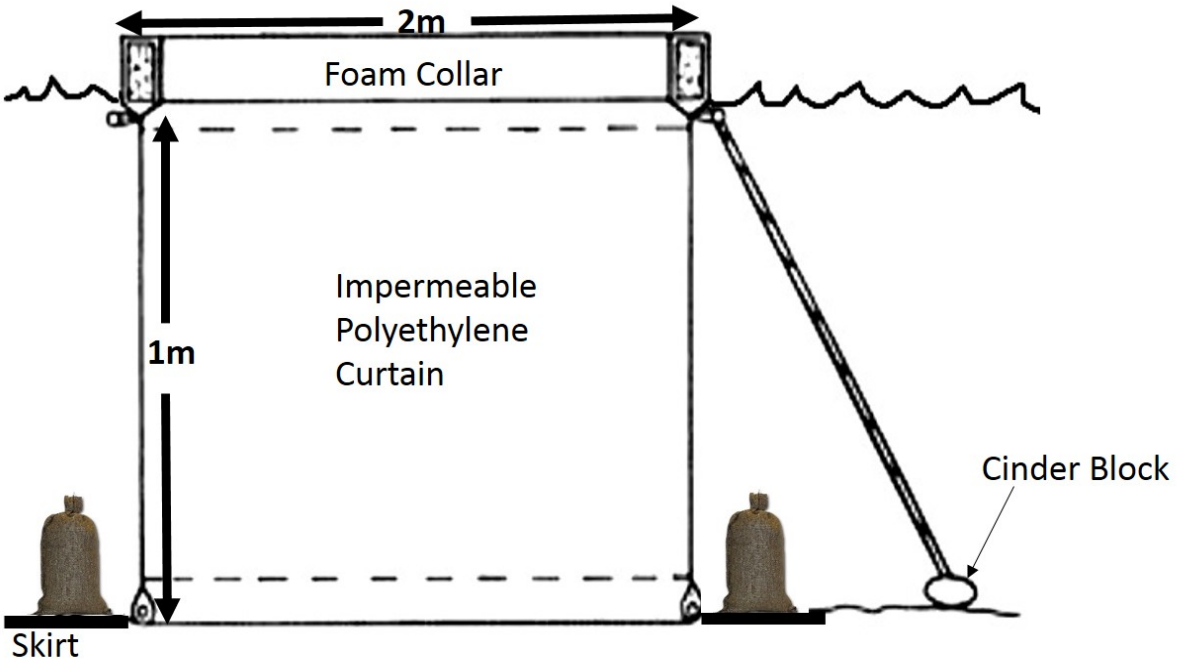

Figure 2.2. Mesocosm Structure. Adapted from Currie Industries (http://www.curryindustries.com/limnocorrals.html)

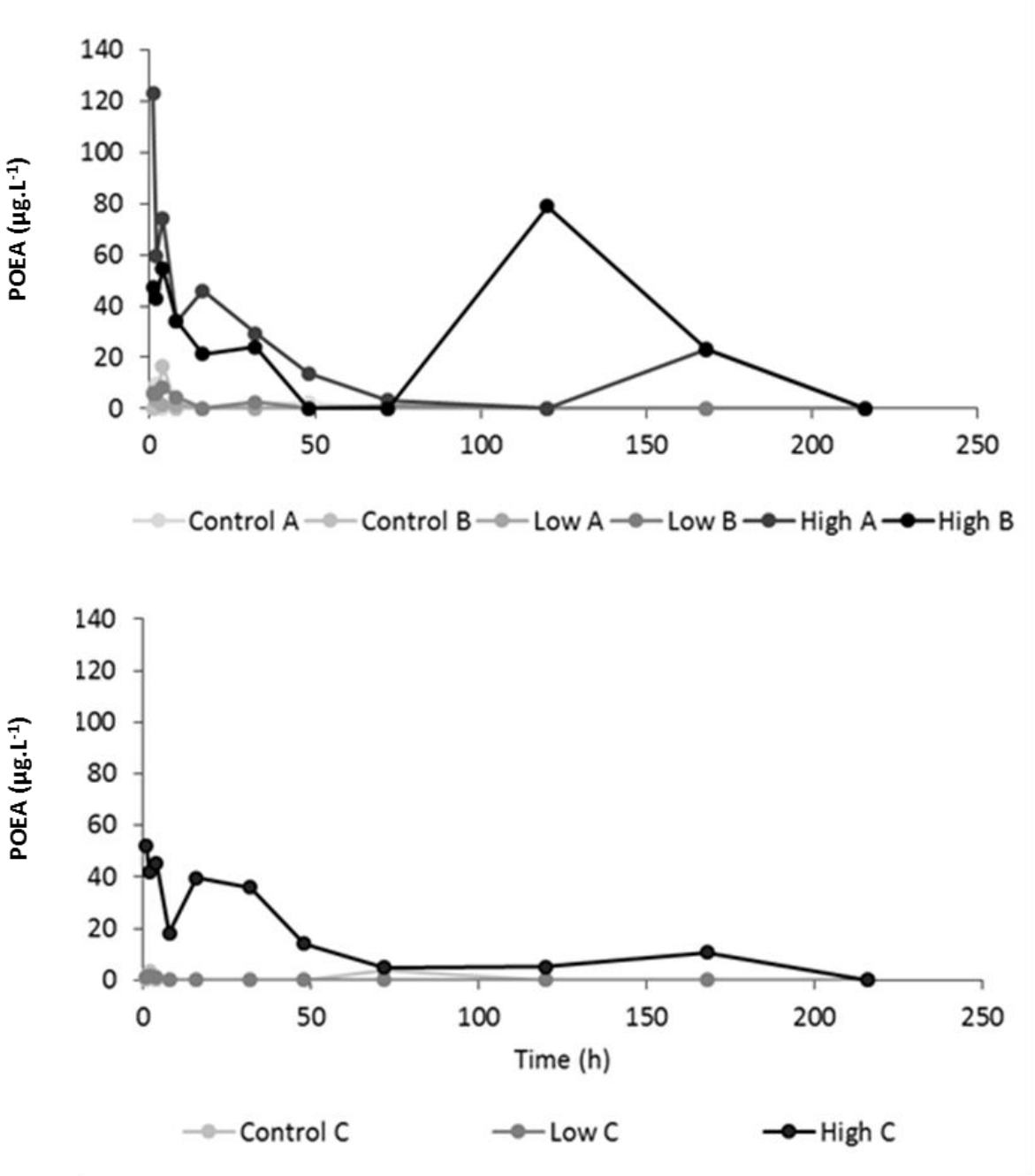

Figure 2.3. POEA concentrations (µg.L^{-1}) over time in water column of open-bottom (top) and closed-bottom mesocosms (bottom). Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 µg.L^{-1} POEA; Low C refers to closed-bottom designed mesocosms treated with 10 µg.L^{-1} POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 µg.L^{-1} POEA; High C refers to closed-bottom designed mesocosms treated with 100 µg.L^{-1} POEA.

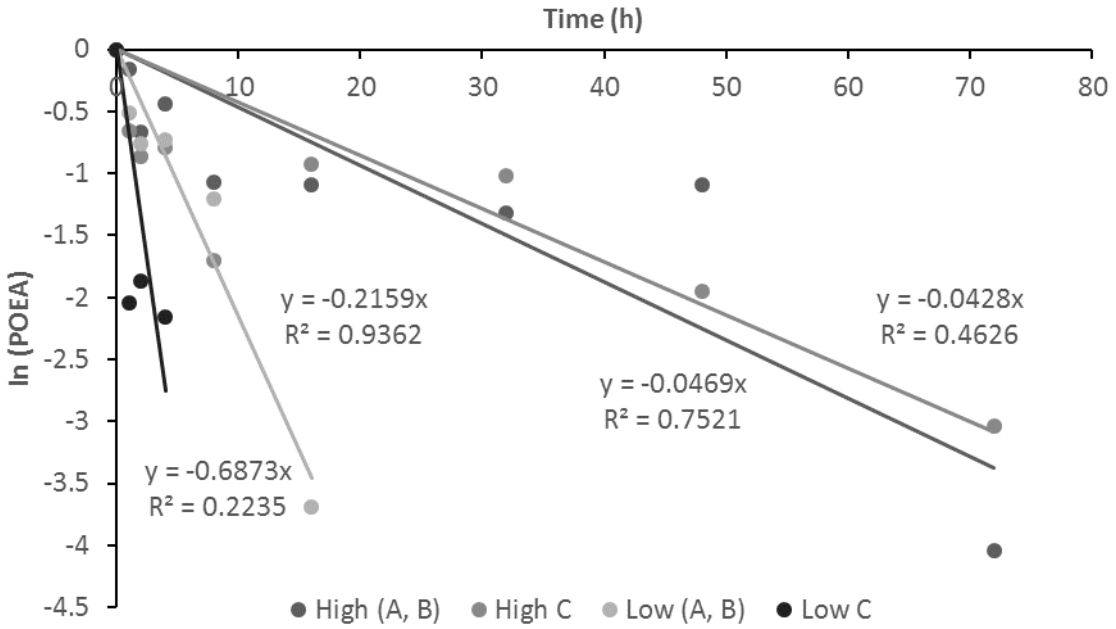

Figure 2.4. DT50 of POEA in the water column of open-bottom mesocosms (mean) and in closed-bottom mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 μg.L^{-1} POEA; Low C refers to closed-bottom designed mesocosms treated with 10 μg.L^{-1} POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 μg.L^{-1} POEA; High C refers to closed-bottom designed mesocosms treated with 100 μg.L^{-1} POEA. Low A and B mean DT50 = 3.2 hrs, Low C DT50 = 1 hr. High A and B mean DT50 = 14.8 hrs, High C DT50 = 16.2 hrs.

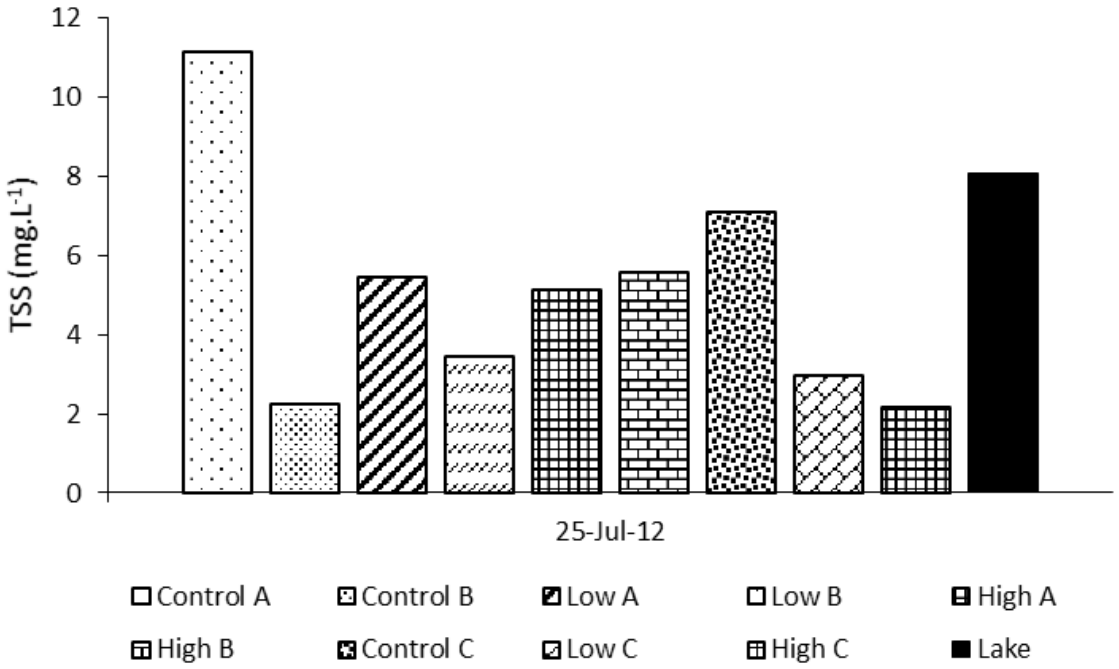

Figure 2.5. TSS ($mg.L^{-1}$) in the water column of each mesocosm on July 25, 2012, one day post POEA treatment. Corresponding DT50s of POEA in the water column of each mesocosm provided within bar. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA.

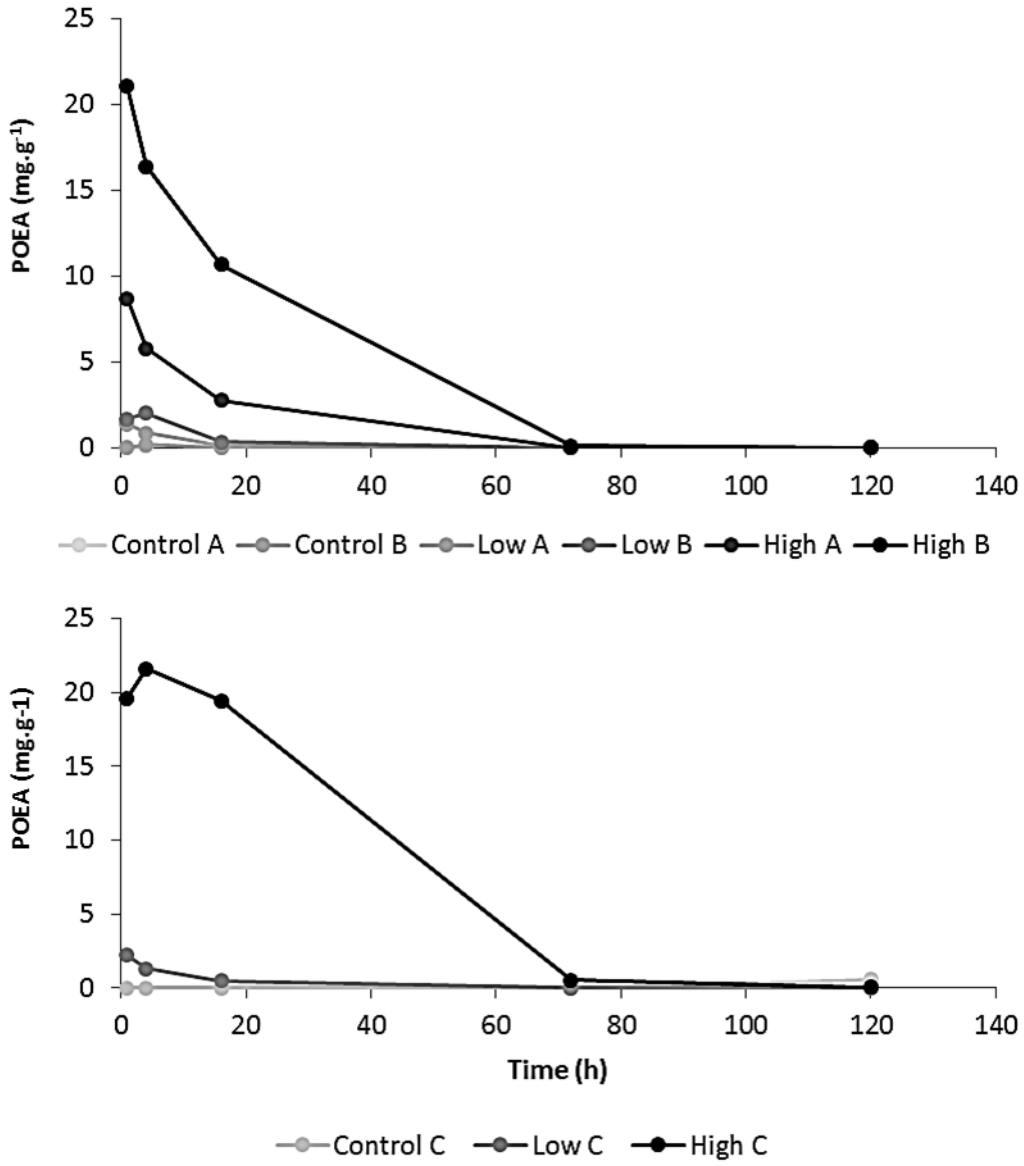

Figure 2.6. POEA concentrations ($mg.g^{-1}$) over time bound to suspended particulates of open-bottom (top) and closed-bottom mesocosms (bottom). Control A and B refer to duplicate treatments of open-bottom designed mesocosms; Control C refers to treatments of closed-bottom bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA; Low C refers to closed-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA; High C refers to closed-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA.

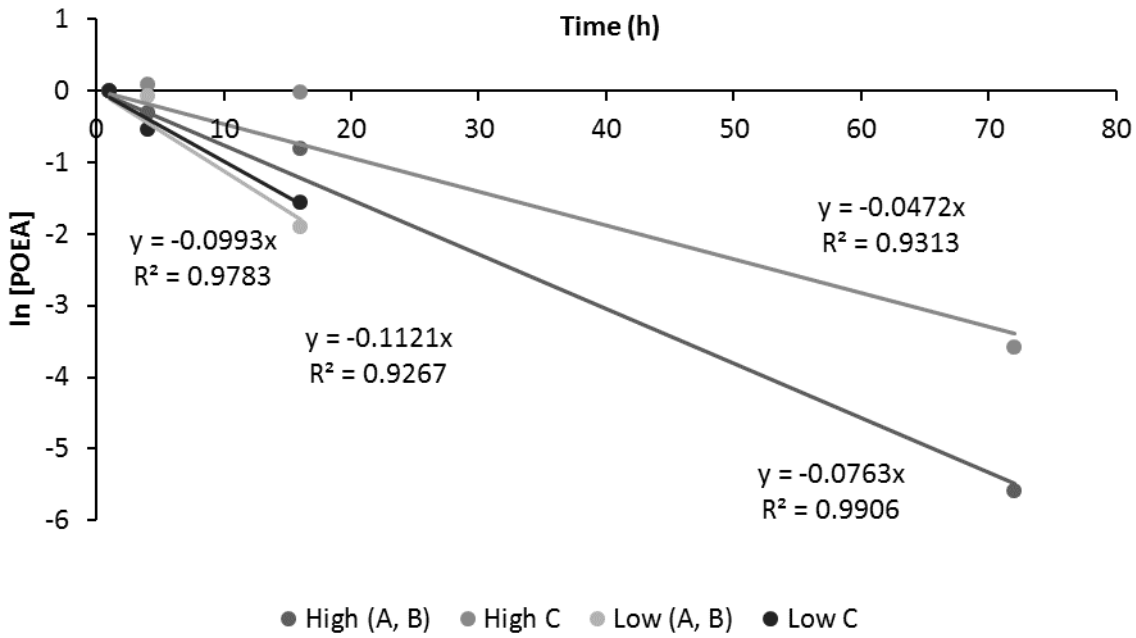

Figure 2.7. DT50 of POEA bound to particulates in open-bottom mesocosms (mean) and in closed-bottom mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 μg.L^{-1} POEA; Low C refers to closed-bottom designed mesocosms treated with 10 μg.L^{-1} POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 μg.L^{-1} POEA; High C refers to closed-bottom designed mesocosms treated with 100 μg.L^{-1} POEA. Low A and B mean DT50 = 5.9 hrs, Low C DT50 = 7.0 hrs. High A and B mean DT50 = 9.1 hrs, High C DT50 = 14.7 hrs.

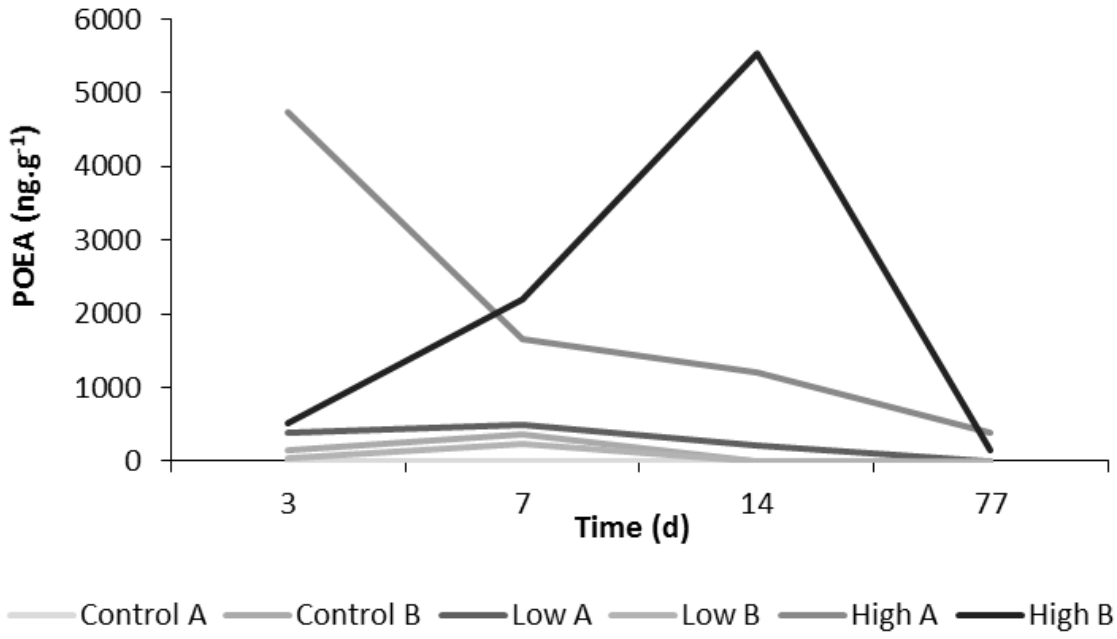

Figure 2.8. POEA concentrations ($ng.g^{-1}$) over time in sediment of open-bottom mesocosms. Control A and B refer to duplicate treatments of open-bottom designed mesocosms. Low A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 10 $\mu g.L^{-1}$ POEA. High A and B refer to duplicate treatments of open-bottom designed mesocosms treated with 100 $\mu g.L^{-1}$ POEA.

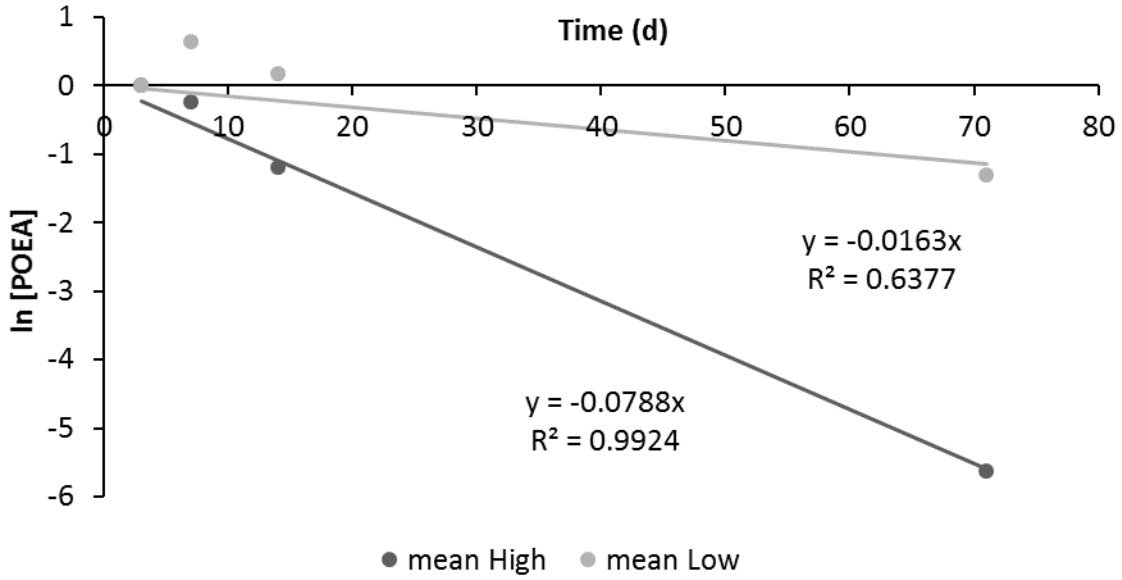

Figure 2.9. Mean POEA DT50 in sediments of open-bottom mesocosms. Low mesocosms treated with 10 μg.L^{-1} POEA. High refers to mesocosms treated with 100 μg.L^{-1} POEA. Mean Low DT50 = 8.8 days. Mean High DT50 = 42.5 days.

Chapter 3: Histolopathological and Biochemical Effects of POEA on *Pimephales promelas*

3.1. Introduction

Polyethoxylated tallow amine (POEA), the surfactant used in the herbicide Roundup® (active ingredient: glyphosate), may be toxic to select aquatic organisms. POEA is a non-ionic surfactant added to herbicide formulations to increase foliar uptake of the active ingredients by penetrating the waxy cuticle of plants (Brausch & Smith, 2007). Studies have shown that POEA is more acutely toxic than glyphosate alone or Roundup® in full formulation (Bradberry et al., 2004).

Exposure of aquatic organisms to environmental pollutants can often induce alterations at the cellular and biochemical levels (Parvez & Raisuddin, 2005). A subchronic exposure found that Roundup® altered normal histology of the gills and liver of fish (Hued et al., 2012). Fish exposed to high doses of Roundup® exhibited a reduction in gill surface area, the portion available for gas exchange (PAGE). Other lesions observed by Hued et al. (2012) were epithelial lifting, hyperplasia/hypertrophy, and fusion of the secondary lamellae. The swelling and proliferation of mucous cells have also been observed in the gills of fish exposed to pollutants (Pereira et al., 2012). Presumably mucous secretion by the cells acts as a protective barrier against toxins. Furthermore, inhibition of cellular transport proteins may result in hypertrophy of cells. Na^+/K^+-ATPase is a key enzyme found in most animal cells and is responsible for the transport of sodium out of the cell and potassium into the cell. In fish gills, Na^+/K^+-ATPase regulates cellular volume, osmotic pressure, and membrane permeability (Sancho et al., 2003).

Damage to the cellular morphology of livers has also been noted in fish species under stressful conditions. Jiraungkoorskul et al. (2003) noted the swelling of hepatocytes and vacuole formation in fish chronically exposed to Roundup®. Such alterations in tissues may be caused by the formation of reactive oxygen species, which due to their highly reactive nature, may damage cells or organelles and thus influence enzyme function and cause lipid peroxidation (Ahmad et al., 2000). Indeed, lipid peroxidation induced by exposure to pollutants has been documented in a number of studies (Schlenk et al., 1997; Sevgileret al., 2004, Glusczak et al., 2007). An increased formation of TBARS can be used to measure the level of oxidative damage to lipid membranes in an organism. Histological lesions in gills and liver, change in brachial Na^+/K^+-ATPase activities, and hepatic lipid peroxidation, have been observed in several fish species exposed to herbicides. (Assem et al., 1995; Glusczak et al., 2006; Glusczak et al., 2007; Hued et al., 2012).

To investigate the effects of short-term POEA exposure on cellular function and influence on select enzyme activities, Fathead minnows (*Pimephales promelas*) were exposed to environmentally relevant concentrations of POEA (Giesy et al., 2000) for representative exposure durations under controlled laboratory conditions. The Fathead minnow was chosen because it is a native freshwater species of ecological importance in North America and it compliments previous field based experiments conducted in this thesis.

3.2. Materials and Methods

Fathead minnows are widely distributed in North America and are frequently used in toxicity studies (Ankley & Villeneuve 2006). Of the 168 Fathead minnows used in the laboratory exposure 9% were juveniles, 22% were adult males, and 69% were adult females. Two hundred individuals were obtained from the FWI (Winnipeg, MB). Fish were transported in coolers to the University of Manitoba animal holding facility in the Duff Roblin building.

Duration of transportation and specimen counting totaled approximately 30 minutes. Fish were then transferred to experimental tanks and acclimated to laboratory conditions for 17 days before the experiment commenced. All experimental fish were maintained following the US EPA (Denny 1988) guidelines in 10 L aerated glass aquarium at 20 ± 2 °C and under a light:dark cycle of 16:8 hours. Dissolved oxygen, aquarium pH, ammonia, nitrite, and nitrate were monitored throughout the experimental period. During the acclimation period and throughout the exposure, fish were fed every second day with commercial fish flakes at a ration of 3% body weight. All described procedures were conducted under approved animal care protocol F13-030 at the University of Manitoba, following the guidelines of the Canadian Council for Animal Care. No mortalities were observed during the acclimation and experimental period.

3.2.1. Acute toxicity test

To test the toxicity of POEA, four groups of six individuals were exposed to one of three POEA treatments: 0 $\mu g.L^{-1}$ (control), 10 $\mu g.L^{-1}$ (low), 100 $\mu g.L^{-1}$ (high) for one week between October 21 and 27, 2013. Prior to exposures, each tank contained 14 fish where males, females and juveniles were randomly apportioned. Two fish from each tank were lethally sampled pre-POEA treatmet using an overdose (0.1 $g.L^{-1}$) of pH buffered (pH = 7.0) tricaine methanesulfonate (MS222) anesthetic. After gill movement ceased (< 5 min) fish were weighed and measured (total length). Mean body mass was 2.3 ± 0.5 g and mean total length was 5.8 ± 0.5 cm (means ± SD) of pre-treatment fish. Gill and liver tissue was removed from each individual. All gill filaments on the right-hand side of the fish and approximately half of the liver was placed in 10% buffered formalin for fixation and subsequent histological analysis. Gill filaments on the left-hand side and the remaining half of the liver were snap frozen in liquid nitrogen and stored at -80 °C for future analysis of

Na+/K+-ATPase activity in gills and lipid peroxidation in livers. Twelve fish remained in the tank until the second day of exposure when six were lethally sampled. The remaining six were sampled on the seventh day (Table 3.1). Fish from both the second and seventh day sample points were processed in the same manner as the pre-exposure specimens.

A 50 L stock solution of both 10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$ POEA was prepared at the same time and stored in clear glass tanks under the same conditions as the treated aquaria. This was to ensure POEA degradation was the same in both the exposure and stock solutions. After the second day of sampling, 5 L of water from the treated aquaria was removed and replaced with 5 L from the corresponding stock solution. This static renewal occurred once more on the fifth day and was followed by final sampling on the seventh day of the experimental period (see Table 3.1 for sampling and exposure protocol). Equivalent water changes were conducted in the control tanks with aquarium water.

3.2.2. Water quality parameters

Temperature, dissolved oxygen (DO), and pH were monitored daily immediately prior to water changes using a handheld YSI multi-probe (Pro Plus, YSI, Yellow Springs, Ohio, USA), in each tank throughout the duration of the POEA exposure. Water samples (20 ml) were taken concurrently and analyzed for ammonia (NH_4^+), nitrate (NO_3), and nitrite (NO_2) using API freshwater test kits. Unionized ammonia (NH_3) was calculated based on NH_4^+ concentration, temperature, and pH (FDEP 2001) in each tank.

3.2.3. Fish condition

The condition of Fathead minnows exposed to POEA was determined using Fulton's condition factor (FCF) as described in Section 2.2.7.6.

3.2.4. Histological sample preparation and biochemical analysis

Gill and liver tissue, freshly dissected from lethally sampled Fathead minnows, was immediately immersed for 24 hours in 10% buffered formalin (pH = 7.2) and stored at room temperature. All observations and numerations were conducted in a blind fashion.

Tissue samples fixed in formalin, were washed three times for 15 minute intervals in tap water and dehydrated for 30 minutes through a graded ethanol series of 70, 80, 95, and 100%. The final step was immersion in Slide Brite™ for clearing and subsequent embedding in paraffin wax. Paraffin embedded wax tissue sections (5 µm) of both gill and liver samples were taken using a Shandon AS325 retraction microtome (serial # MC9501208) and mounted on glass slides prior to staining with Hematoxylin and Eosin (H & E) to stain nuclei blue and cytoplasm pink. It was determined that three minutes in hematoxylin, five minutes in the destaining solution (acid alcohol), and 3 minutes in eosin was best suited for the formalin preserved gill and liver tissue.

Gills were also stained using the Periodic Acid-Schiff (PAS) technique, which reveals carbohydrate compounds. In this case, the carbohydrate compounds were glycoproteins produced by mucous cells. Following paraffin removal, slides were stained for 5 min in periodic acid, flushed for 5 min with dH_2O, soaked for 10 min in Schiff's reagent, bleached for 2 min, and flushed again in distilled water for 10 min prior to dehydrating and mounting. The PAS stain was used to examine gills for proliferation of mucus cells (Pereira et al. 2009).

Slides were examined using an Olympus BX43 Upright Light Microscope at 40X objective magnification and photographed using digital imaging software (Infinity 1 by Lumenera Corporation).

3.2.5. Gill histopathology

Gill morphometric analysis was performed based on modified methods described by Nero et al. (2006) and Hued et al. (2012). For statistical rigor, ten gill filaments were selected and examined for each individual. A central section of five secondary lamellae on each side of the gill filament were selected for measurement (Figure 3.1). The basal epithelial thickness (BET) was measured on both sides of the blood sinus at three points (top, middle and bottom) of the selected section. The secondary lamellae length (SLL) was measured from the distal end to the base of each of the 10 lamellae in the section. The measured parameters denote gas diffusion distance in fish gills (Hued et al., 2012). The portion of gills available for gas exchange (PAGE) was calculated using the following formula: %PAGE= 100*{mean SLL / (2*mean BET + mean SLL)}. The PAGE values were calculated for each filament and averaged for each individual.

To examine gills for the proliferation of mucous cells, similarly to the methods described for determining the PAGE, mucous cells were enumerated for the central section of five secondary lamellae on either side of the primary lamellae in ten filaments for each individual.

3.2.6. Na^+/K^+-ATPase activity

For measurement of Na^+/K^+-ATPase activity, the entire gill mass of one side of each Fathead minnow was used. Flash frozen gill filaments were thawed and homogenized in ice cold 600 µL SEID homogenization buffer: (150 mM sucrose, 10 mM EDTA, 0.1% NaDeoxycholate, 50 mM imidazole) stored for a maximum of 4 days at 4 °C (modified from Gibbs & Somera 1990, Mc Cormick 1993). Eight gill samples were homogenized per assay immediately prior to analysis, using a TissueLyser (Qiagen, Toronto, ON, Canada) set to 30 rpm for 90 seconds. Homogenates were then centrifuged at 4 °C for one minute at 5000 g (Accuspin, Thermo Fisher, Mississagua, On, Canada) and the supernatant was transferred into a snap-cap vial and

stored on ice. Excess homogenate was stored at -80 °C and subsequently used to determine total protein using a Bradford assay (BioRad).

The Na^+/K^+-ATPase assay reagents were prepared daily and stored protected from light (modified from Gibbs & Somera, 1990; McCormick 1993). The assay mixture contained 100 mM NaCl, 5 mM $MgSO_4$, 50 mM imidazole, 3 mM ATP, 2 mM PEP, 0.2 mM NADH, 5 IU/ml pyruvate kinase, and 4 IU/ml LDH. A second assay mixture was made up as above but also contained 5.5mM Oubain. An ion substrate (1 M KCl) was prepared as needed and stored at 4 °C.

Gill homogenate samples were measured for Na^+/K^+-ATPase activity using a spectrophotometer (Agilent Technologies Cary Series 100, SN: MY12320002, wavelength: 340 nm, duration: 15 min) at 20 °C to match POEA exposure temperature. To determine total ATPase activity, 40 µL of homogenate, 10 µl of ion substrate, 50 µl dH2O, and 900 µl of total ATPase solution was added to 1.5 ml disposable cuvettes in triplicate. To determine Na^+/K^+-ATPase activity inhibition, 40 µL of homogenate, 10 µl of ion substrate, 100 µl dH2O, and 900 µl of 5.5 mM Oubain was added to 1.5 ml disposable cuvettes in triplicate. The difference between the total ATPase activity and the ATPase activity measured in the presence of the Na^+/K^+-ATPase specific inhibitor Oubain was calculated to establish the fraction of activity which was due explicitly to Na^+/K^+-ATPase.

To ensure that no loss of total Na^+/K^+-ATPase activity occurred, the sample homogenate and Na^+/K^+-ATPase reagents were stored separately and combined only immediately prior to analysis. Protein content of the gill homogenate was measured using the Bio-Rad Quick Start Bradford protein microassay using bovine serum albumin (BSA) as a standard. To safeguard that homogenate protein concentrations fell within the linear range of BSA standards of the assay (1.25 – 10 $\mu g.ml^{-1}$), samples were diluted 1:256. This dilution factor was determined

through a series of dilutions and pilot assays. At the final dilution factor, there was no occurrence of absorbance interference caused by the SEID homogenization buffer. The protein concentration of the gill homogenates were measured in triplicate using a FLUOROstar Omega plate reader (SN: 415-1572). Na^+/K^+-ATPase activity was expressed as $\Delta\mu mol\ substrate.hr^{-1}.mg\ protein^{-1}$ for each sample.

3.2.7. Liver histopathology

Liver samples of specimens from each treatment were inspected for swelling of hepatocytes and vacuole formation. The hepatocyte volume index was determined for each sample by enumerating the number of hepatocytes observed in a 0.16 mm^2 field-of-view (FOV) at 40X objective magnification.

3.2.8. TBARS formation

Lipid peroxidation in liver samples was estimated from the formation of TBARS, an end product of lipid peroxidation. Thiobarbituric acid reacts with malondialdehyde (MDA) to yield a fluorescent product (Trevisan et al., 2001). For measurement of TBARS, half of the liver mass of each exposed Fathead minnow was used. Flash frozen livers were thawed, weighed and re-suspended at 50 $mg.ml^{-1}$ in normal saline. If samples were less than 5 mg, they were pooled with another sample from the same experimental tank. Twenty-four liver samples were homogenized per assay immediately prior to analysis, using a TissueLyzer set to 30 rpm for 60 seconds. Whole homogenate was analyzed using the TBARS assay according to the manufacturer's instructions (Enzo Life Sciences, OXItek, Catalog#: ALX-850-287). To safeguard that sample TBARS concentration fell within the linear range of the assay standards, samples were diluted 1:10. All reagents and standards were prepared fresh for each analysis. The standards were prepared for fluorometric analysis by diluting the concentrated standard provided in the assay kit. Linear standards (0 – 40 nmol/ml) were

prepared following the table provided in the manufacturer's instructions. To accommodate the small volumes of sample, test procedure volumes were reduced by half. Only 50 µl of sample or standard was added to a disposable glass test tube with 50 µl sodium dodecyl sulfate (SDS) solution and 1.25 ml TBA/Buffer reagent. Aside from modifications of sample and reagent volumes, the test procedure was followed as described in the manufacturer's instructions. The tubes were stopped with a glass marble and incubated at 95 °C for 60 min. Following incubation, samples were placed in an ice bath and cooled to room temperature and then centrifuged (Thermo Scientrific Multifuge X3R, SN: 4095325B) at 3000 rpm for 15 min. The supernatant was removed and measured in triplicate using a fluorimeter (FLUOROstar Omega, excitation: 530 nm, emission: 550 nm, sensitivity: high, slit width: 5 nm).

The protein content of the liver homogenate was measured as described in Section 3.2.6 with an applied dilution factor of 1:600, TBARS was expressed as nmol.mg protein^{-1} in each sample.

3.2.9. Statistical Analysis

Data distributions were analyzed using a univariate analysis of the residuals and the Shapiro-Wilk test for normality. Data were considered normally distributed if $p < 0.05$. All biological parameters that were not distributed normally were transformed accordingly. Datasets which could not be transformed and violated the assumptions of parametric analysis were examined using non-parametric tests. Differences were considered significant at $p < 0.05$.

All statistical analyses of data were performed in SAS 9.4. The data are presented as means with standard deviations of the mean (mean ± SD).

3.2.9.1. Determining tank effect

A one-way analysis of variance (ANOVA) was performed on all specimens sampled from the 7 day control exposures to examine tank effect in all histological and biochemical tests.

3.2.9.2. POEA effect on gills

To examine differences in the PAGE measurements, mucous cell counts, and Na^+/K^+-ATPase activity in gills of Fathead minnows between POEA exposure duration and treatment concentration, a two-way ANOVA was performed on normally distributed datasets. If significant differences were observed, a one-way ANOVA was performed separately on 2 and 7 day exposure treatments. If data were not normally distributed, and could not be transformed, a Kruskal-Wallis non-parametric test was performed. Significant differences observed in both parametric and non-parametric tests were followed by a Bonferroni or Scheffe post hoc to identify differences between specific pairs.

3.2.9.3. POEA effect on livers

The same statistical methodology as described in Section 3.2.9.2 was followed to examine differences in the hepatocyte counts and TBARS formation in livers of Fathead minnows between POEA exposure duration and treatment concentration.

3.3. Results

3.3.1. Water quality parameters

Daily monitoring results of water quality parameters in each tank throughout the duration of the experiment are documented in Appendix E. Over the 7 day experiment, temperature in the control tanks was 18.0 ± 0.71 °C, 18.0 ± 0.85 °C in the low treatment tanks, and 17.4 ± 0.90

°C in the high treatment tanks (Figure 3.2). Dissolved oxygen in the control tanks was 7.8 ± 0.35 $mg.ml^{-1}$, 7.6 ± 0.41 $mg.ml^{-1}$ in the low treatment tanks, and 7.9 ± 0.38 $mg.ml^{-1}$ in high treatment tanks. The pH in the control tanks was 7.8 ± 0.13, 7.6 ± 0.23 in the low treatment tanks, and 7.8 ± 0.21 in high treatment tanks. There was an increasing trend in unionized ammonia in all tanks throughout the exposure. The NH_3 levels in the control tanks was 0.037 ± 0.021 $mg.L^{-1}$, 0.046 ± 0.023 $mg.L^{-1}$ in the low treatment tanks, and 0.039 ± 0.018 $mg.L^{-1}$ in high treatment tanks throughout the POEA exposure period. There was no statistically significant difference between exposure treatments for each of the water quality parameters monitored.

3.3.2. Determination of tank effect

There were no evident tank effects for gill or liver histology.

3.3.3. Acute toxicity test

All physiological data for fish in the POEA laboratory exposure from October 21 – 27, 2013 are compiled in Table 3.2 and Appendix F.

3.3.4. POEA effect on fish condition

Fulton's condition factor (FCF) was significantly greater among fatheads on the second day of POEA exposure, than the seventh day (Table 3.2). The two-way ANOVA, showed a significant difference ($p = 0.003$) in the mean FCF in Fathead minnows. One-way ANOVAs performed separately on second and seventh day exposures revealed no significant differences ($p = 0.318$ and $p = 0.123$, respectively) between the FCF calculated in specimens sampled from each of the POEA treatment concentrations (control, low, high).

3.3.5. POEA effects on gills

Histopathological alterations to Fathead minnow gills caused by exposure to POEA included epithelial lift, fusion of secondary lamellae, hyperplasia, and epithelial rupture (Figure 3.3).

3.3.5.1. PAGE

The PAGE did not differ in pre-exposure fish (30.5 – 54.9 %) versus the reference fish on the second day (26.0 – 52.8 %) and seventh day (20.4 – 56.5 %) (Table 3.2, Figure 3.4). The two-way ANOVA results revealed no significant difference between the exposure duration, the treatment, nor the interaction of the two ($p = 0.995$, $p = 0.998$, $p = 0.073$, respectively). One-way ANOVAs performed separately on PAGE values from fish on the second and seventh day of exposure revealed no significant differences ($p = 0.357$ and $p = 0.165$, respectively) between the PAGE percentages calculated in gills of specimens sampled from each of the POEA treatment concentrations (control, low, high).

3.3.5.2. Mucous cell counts

POEA had an effect on mucous cell proliferation. The mucous cell counts in Fathead minnow gills pre-POEA exposure ranged from 15 – 26 (21 ± 2.8) (Table 3.2, Figure 3.5, Figure 3.6). In comparison, the mucous cell counts in Fathead minnow gills from the control tanks on the second and seventh day of exposure ranged from 10 – 23 (17 ± 4.0) and 11 – 25 (20 ± 3.3), respectively. In the low treatment, the mucous cell counts ranged from 12 – 28 (22 ± 6.1) and 20 – 34 (25 ± 5.5) on the second and seventh day of exposure, respectively. In the high treatment, the mucous cell counts ranged from 15– 26 (19 ± 3.7) and 20 – 41 (30 ± 7.2) on the second and seventh day of exposure, respectively. The two-way ANOVA results showed significant differences in the duration and treatment concentrations of POEA ($p < 0.001$ and $p = 0.005$, respectively). The Bonferroni pairwise comparison identified significant differences between specific pairs (Table 3.3). Fathead minnows exposed to 100 $\mu g.L^{-1}$

POEA for 7 days had a significantly greater mean number of mucous cells than fish in all of the control exposures ($p < 0.001$). Fish sampled on the seventh day from the high POEA treatment exposure were also determined to have a significantly greater number of mucous cells than fish in the low and high POEA treatments for 2 days ($p < 0.001$ and $p = 0.009$, respectively). Fathead minnows exposed to 10 $\mu g.L^{-1}$ POEA for 7 days had a significantly greater mean number of mucous cells than fish in the control tanks on the second day.

3.3.5.3. Na^+/K^+-ATPase

POEA affected Na^+/K^+-ATPase in a transient manner. The Na^+/K^+-ATPase activity in Fathead minnow gills pre-POEA exposure ranged from 0.13 – 5.86 (1.44 ± 1.19 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) (Table 3.2, Figure 3.7). In comparison, the Na^+/K^+-ATPase activity in Fathead minnow gills from the control tanks on the second and seventh day of exposure ranged from 0.11 – 4.63 (1.22 ± 1.10 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) and 0.59 – 4.05 (1.94 ± 0.97 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$), respectively. In the low treatment, the Na^+/K^+-ATPase activity in gills ranged from 0.14 – 1.91 (1.01 ± 0.46 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) and 0.31 – 6.18 (2.02 ± 1.65 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) on the second and seventh day of exposure, respectively. In the high treatment, the Na^+/K^+-ATPase activity ranged from 0.06 – 1.40 (0.72 ± 0.41 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) and 0.69 – 4.87 (2.09 ± 0.97 $\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) on the second and seventh day of exposure, respectively. Na^+/K^+-ATPase

The Na^+/K^+-ATPase activity in Fathead minnow gills declined slightly from pre-exposure and control levels after the second day of exposure to 10 $\mu g.L^{-1}$ POEA, and considerably after the second day of exposure to 100 $\mu g.L^{-1}$ POEA. By the seventh day of exposure to both POEA treatments, Na^+/K^+-ATPase activity levels were relatively equal to those observed in the gills of control specimens. The Kruskal-Wallis test determined significant differences ($p < 0.001$) between the mean Na^+/K^+-ATPase activity in gills of

specimens sampled from each of the POEA treatment exposures. The Scheffe multiple comparison post hoc test revealed that mean Na^+/K^+-ATPase activity in gills on the second day of high exposure was significantly lower than observed on the seventh day of fish sampled from the control, low, and high POEA treatments.

3.3.6. POEA effects on liver

Slides with liver tissue sections that were noted to be too compressed, or too thick were not included in data analysis (n = 3).

3.3.6.1. Hepatocyte counts

POEA had no significant effect on hepatocyte volume. Hepatocyte counts are expressed per FOV (0.16 mm^2). The hepatocyte counts in Fathead minnow livers pre-POEA exposure ranged from 343 – 695 (503 ± 85.6) (Table 3.2, Figure 3.8, Figure 3.9). In comparison, the hepatocyte counts in Fathead minnow livers from the control tanks on the second and seventh day of exposure ranged from 276 – 857 (506 ± 166.2) and 351 – 646 (484 ± 105.1), respectively. In the low treatment, the hepatocyte counts ranged from 428 – 810 (579 ± 130.7) and 362 – 733 (552 ± 135.6) on the second and seventh day of exposure, respectively. In the high treatment, the hepatocyte counts ranged from 357– 707 (519 ± 132.1) and 390 – 633 (484 ± 107.2) on the second and seventh day of exposure, respectively. The Kruskal-Wallis test revealed no significant differences ($p = 0.743$) between the mean hepatocyte counts in the liver of specimens sampled from each of the POEA treatment exposures (pre-exposure; 2 day control, low, high; 7 day control, low, high).

3.3.6.2. TBARS formation

Mean TBARS levels doubled in Fathead minnow livers exposed to 10 $\mu g.L^{-1}$ POEA for 7 days as compared to mean levels observed in the control specimens. The TBARS

produced in Fathead minnow liver pre-POEA exposure ranged from 0.60 – 28.91 (11.59 ± 9.30 nmol.mg protein^{-1}) (Table 3.2, Figure 3.10). In comparison, the TBARS produced in Fathead minnow liver from the control tanks on the second and seventh day of exposure ranged from 0.66 – 22.63 (11.72 ± 6.98 nmol.mg protein^{-1}) and 0.72 – 64.16 (14.92 ± 18.53 nmol.mg protein^{-1}), respectively. In the low treatment, TBARS formation ranged from 0.61 – 30.37 (12.90 ± 9.04 nmol.mg protein^{-1}) and 0.43 – 120.85 (28.77 ± 35.49 nmol.mg protein^{-1}) on the second and seventh day of exposure, respectively. In the high treatment, TBARS formation ranged from 0.52 – 28.79 (11.81 ± 7.61 nmol.mg protein^{-1}) and 0.91 – 70.18 (14.93 ± 18.28 nmol.mg protein^{-1}) on the second and seventh day of exposure, respectively. The Kruskal-Wallis test revealed no significant differences ($p = 0.927$) between the mean TBARS levels in the liver of specimens sampled from each of the POEA treatment exposures.

3.4. Discussion

Fish gills play an important role in gas exchange (respiration) and ion exchange. Their exterior location and large surface area make them a primary target of environmental pollutants (Ballesteros et al. 2007; Ayoola 2008; Albinati et al. 2009, Hued et al., 2012). The diffusion of dissolved oxygen through the gills of fish is a process affected by the membrane surface area available. A reduction in gill surface area would lead to the inhibition of maintaining a continuous oxygen / carbon dioxide concentration gradient in the circulatory system of fish (Randall et al., 1967; Hughes, 1972). The mean percent PAGE calculated pre-exposure as well as in the control tanks after both 2 and 7 days in the POEA acute toxicity study were 20 – 25 % lower than values reported by Hued et al. (2012) in *Jenynsia multidentata* following a 7 day subchronic toxicity test to 0.5 mg.L^{-1} Roundup® (64.9 ± 6.5). Although control values were lower, a similar decrease in the mean PAGE percentage on the second day of exposure to 10 µg.L^{-1} and 100 µg.L^{-1} POEA were observed. In the current

study, the mean PAGE percentage decreased by 6%, which is comparable to the 10 % decrease observed in the Hued et al. study. The plastic morphology of gill structure has been documented in a number of studies (Ong et al., 2006; Sollid et al., 2007; Cerqueira et al., 2011; Nilsson et al., 2012), and as predicted, due to the short DT50 of POEA in the water column, the morphology of gills in Fathead minnows exposed to POEA for 7 days resembled gills from fish in control tanks. Following the 7 day POEA exposure, the PAGE calculated in Fathead minnow gills also returned to values near those observed in the control and pre-exposure specimens (Section 3.3.5.1).

Further, complications of gas exchange in the gills is observed through the proliferation of mucous cells. Mucous cells secrete fluid rich in glycoproteins, which serve to protect against the intake of pollutants (Reese et al., 2011; Pereira et al., 2012). As a defense mechanism, the single layer epithelium of the gills becomes multi-layered and in the process many of these cells are converted to mucous cells. The result is an increase in mucus secretion, which makes gas exchange more difficult (Schäperclaus et al., 2011). Although only a subtle increase in mucous cells on the second day of exposure to 10 $\mu g.L^{-1}$ POEA was observed, following the seventh day of exposure to 10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$ POEA, the proliferation in mucous cells was 25 % and 50% greater, respectively. It is possible that this compensatory response may be more delayed and take longer to recover than morphological responses in gills such as the shortening of secondary lamellae and thickening of basal lamellae demonstrated in the PAGE analysis.

Along with gas exchange, gill function also includes ionic regulation, acid-base balance, and nitrogenous waste excretion; processes controlled by active and passive transport of dissolved ions through cellular membranes (Evans, 1987). Environmental pollutants usually reduce Na^+/K^+-ATPase activity (Haya et al., 1983). Results of this study imply that the inhibition of

Na^+/K^+-ATPase activity was influenced by POEA exposure duration and concentration. The Na^+/K^+-ATPase activity levels observed were within the range documented in other studies investigating the effects of environmental pollutants on Fathead minnows (Watson et al., 1987; Peles et al., 2012).

Fathead minnows sampled on the second day of high exposure exhibited Na^+/K^+-ATPase activity levels significantly lower than those observed in the group exposed for 7 days, which exceeded pre-exposure values. In a study conducted by Peles et al. (2012), significant inhibition of Na^+/K^+-ATPase was reported in Fathead minnows following exposure to a sublethal concentration of copper for 24 hrs. All subsequent exposure durations exhibited an increase in Na^+/K^+-ATPase levels from reference values. It may be possible that the greatest inhibition of Na^+/K^+-ATPase induced by POEA exposure occurred prior to the initial sampling time, on the second day. Peles et al. (2012) suggest that elevated Na^+/K^+-ATPase activity likely indicates the increase in the cellular component of this enzyme that is required to rapidly restore ionic balances in exposed fish.

In fish, the liver is a metabolic organ that assists in digestion, storage, and detoxification. Through histological analysis and biochemical assays, Roundup® and/or associated compounds (IPA salt of glyphosate, glyphosate acid, POEA) have been shown to cause pathological damages to the liver (Szarek et al., 2000; Jiraungkoorskul et al., 2003; Gluszak et al., 2007; Hued et al., 2012). In previous studies, histopathological analysis of livers in fish from short-term exposures to 5 $mg.L^{-1}$ Roundup® (750 $\mu g.L^{-1}$ POEA) displayed hydropic degradation (or cellular swelling due to the accumulation of water in the cell) (Jiraungkoorskul et al., 2003; Hued et al., 2012). Prolonged exposure, exceeding 2 months, at this concentration led to mild infiltration of leukocytes (Jiraungkoorskul et al., 2003). In the current study, it was expected that hepatocyte numbers would decrease in each field-of-view

due to an increase in swelling with increased POEA exposure concentration and duration. However, no significant differences in the hepatocyte counts of Fathead minnows between POEA treatments were observed. The SD reported for mean hepatocyte counts in each treatment group was quite high, emphasizing the high variability within the small sample sizes. Small sample sizes poorly define the SD and it is possible that the subset of liver samples analyzed for histopathological alteration were not representative of the true population. As described in Section 3.2.1, only half of the liver was reserved for histological analysis. In some cases, if livers were too small they were preserved exclusively for analysis of biochemical parameters. This complication reduced the total sample size and may have inadvertently caused bias in the subset, which was randomly selected to represent each POEA treatment group.

Lipid peroxidation has been evaluated as a biomarker for oxidative stress in the livers of fish exposed to environmental pollutants in a number of studies (Ahmad et al., 2000; Li et al., 2003; Ajimoko et al., 2007; Gluszak et al., 2007; Liu et al., 2008). In the present study, the TBARS content did not differ significantly between individuals exposed to 10 $\mu g.L^{-1}$ or 100 $\mu g.L^{-1}$ POEA regardless of exposure duration (2 or 7 days) even though mean TBARS levels doubled in Fathead minnow livers exposed to 10 $\mu g.L^{-1}$ POEA for 7 days as compared to mean levels observed in the control specimens after 7 days. In the 2007 study conducted by Gluszak et al. (2007), alterations to TBARS levels were not observed in the livers of Silver catfish (*Rhamdia quelen*) exposed to 0.2 and 0.4 $mg.L^{-1}$ Roundup® (30 and 60 $\mu g.L^{-1}$ POEA). However, Li et al. (2003), reported an increase in TBARS levels in the liver of Crucian carp (*Carassius auratus*) exposed to 3,4-dichloroaniline (0.4 $mg.L^{-1}$) for 15 days. Similarly, Liu et al. (2008) described a 7-fold increase in lipid peroxidation in Zebrafish (*Danio rerio*) exposed to 80 µg perfluorododecanoic $acid.g^{-1}$ bodyweight for 7 days. This suggests that the degree of lipid peroxidation may vary between fish species due to variation in coping mechanisms of

hepatic enzymes to antioxidants (Ahmad et al., 2000). Furthermore, as described for hepatocyte counts, the SD reported for mean TBARS levels in each treatment group was also very high, again highlighting the large degree of variability between samples.

3.5. Conclusion

This experiment showed that environmentally relevant concentrations of the surfactant POEA used in agriculture and forestry industries may cause changes to the histology and biochemical function in the gills of Fathead minnows. Results indicated a proliferation of mucous cells in gills of Fathead minnows exposed to POEA. Na^+/K^+-ATPase activity was significantly reduced on the second day of POEA exposure, but activity exceeded pre-exposure values by the seventh day. Oxidative stress in the liver of Fathead minnows exposed to POEA was not considered significant based on TBARS formation.

Table 3.1. Experimental design and sampling regime for Fathead minnow laboratory POEA exposure

POEA Treatment	Sampling points (days)	# of fish sampled pre-treatment	# of fish sampled / exposure time point	# of replicate tanks	Total # of fish
Control (0 µg.L^{-1})	0, 2, 7	2	6	4	(2+6+6) x 4 = 56
Low (10 µg.L^{-1})	0, 2, 7	2	6	4	(2+6+6) x 4 = 56
High (100 µg.$L^{-1)}$	0, 2, 7	2	6	4	(2+6+6) x 4 = 56
Total		**6**	**18**	**12**	**168**

Table 3.2. Results of POEA effects on histopathology and biochemical parameters of Fathead minnows. (FCF: Fulton's condition factor; PAGE: gill portion available for gas exchange; TBARS: thiobarbituric acid reactive substances. Superscript letters denote significant difference between treatments for a measured variable. Values expressed as mean ± SD, (range). n = 24 in each treatment group with the following exceptions: [1] n = 22, [2] n = 8, [3] n = 21, [4]n = 16, [5]n = 7, [6]n = 11, [7]n = 4, [8]n = 18, [9]n = 15, [10]n = 19, [11]n = 20. * Reduced sample sizes due to pooling within groups.

	Pre-exposure	2 Day Exposure			7 Day Exposure		
		Control	Low	High	Control	Low	High
FCF	1.01 ± 0.19 (0.82 – 1.63)	1.01 ± 0.16 (0.60 – 1.34)	1.05 ± 0.15 (0.80 – 1.34)	0.98 ± 0.13 (0.49 – 1.19)	0.91 ± 0.14 (0.68 – 1.33)	0.99 ± 0.14 (0.74 – 1.30)	0.94 ± 0.12 (0.73 – 1.13)
Gills							
PAGE (%)	42.0 ± 6.8 (30.5 – 54.9)[1]	45.3 ± 8.8 (26.0 – 52.8)[2]	39.1 ± 11.9 (29.7 – 58.5)[2]	38.8 ± 9.1 (24.4 – 49.5)[2]	36.8 ± 9.0 (20.4 – 56.5)	43.2 ± 8.9 (29.2 – 55.6)[2]	43.1 ± 6.9 (31.1 – 50.8)[2]
Mucous cell counts	21 ± 3[acdef] (15 – 26)[3]	17 ± 4[acdef] (10 – 23)[2]	22 ± 6[acdef] (12 – 28)[2]	19 ± 4[acdef] (15 – 26)	20 ± 3[acdef] (11 – 25)	25 ± 5[abdef] (20 – 34)[2]	30 ± 7[b] (20 – 41)[2]
Na^+/K^+-ATPase ($\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$)	1.44 ± 1.19[ab] (0.12 – 5.86)	1.23 ± 1.10[ab] (0.11 – 4.63)	1.01 ± 0.14[ab] (0.14 ± 1.91)	0.72 ± 0.41[a] (0.06 – 1.40)	1.94 ± 0.97 [b] (0.59 – 4.05)	2.02 ± 1.65 [b] (0.31 – 6.18)	2.09 ± 0.97 [b] (0.69 – 4.87)
Gill protein ($mg.ml^{-1)}$	1.04 ± 0.82 (0.15 – 3.70)	1.19 ± 0.88 (0.26 – 3.77)	1.35 ± 0.86 (0.31 – 4.18)	1.39 ± 0.88 (0.26 – 4.33)	0.80 ± 0.47 (0.26 – 2.15)	0.81 ± 0.46 (0.11 – 1.74)	0.87 ± 0.43 (0.26 – 1.84)
Liver							
Hepatocyte counts (per 0.16 mm^2 FOV)	503 ± 86 (343 – 695)[3]	506 ± 166 (276 – 857)[4]	579 ± 131 (428 – 810)[5]	519 ± 132 (357 – 707)[2]	484 ± 105 (351 – 646)[6]	552 ± 136 (362 – 733)[5]	484 ± 107 (390 – 633)[7]
TBARS* (nmol.mg $protein^{-1}$)	11.59 ± 9.30 (0.60 – 28.91)[8]	11.72 ± 6.98 (0.66 – 22.63)[9]	12.90 ± 9.04 (0.61 – 30.37)[8]	11.81 ± 7.61 (0.52 – 28.79)[8]	14.92 ± 18.53 (0.72- 64.16)[4]	28.77 ± 35.49 (0.43 – 120.85)[10]	14.93 ± 18.28 (0.91 - 70.18)[11]
Liver protein ($mg.ml^{-1}$)	5.07 ± 2.02 (2.02 – 10.07)[8]	6.75 ± 1.43 (3.14 – 9.14)[9]	5.65 ± 1.05 (3.80 -7.37)[8]	6.03 ± 1.23 (3.32 – 8.31)[8]	4.98 ± 1.82 (1.41 -7.69)[4]	5.93 ± 2.15 (1.66 – 8.83)[10]	5.99 ± 1.55 (3.49 – 10.02)[11]

Table 3.3. Bonferroni post hoc pairwise comparisons results with significant differences between mucous cell counts in Fathead minnow gills in all POEA exposure treatments.

Pairs	T statistic	p-value
Pre-exposure vs 7 Day High	4.908	0.0001
2 Day Control vs 7 Day Low	3.989	0.0031
2 Day Control vs 7 Day High	5.925	1.68E-06
7 Day Control vs 7 Day High	5.221	3.02E-05
2 Day Low vs 7 Day High	4.869	0.0001
2 Day High vs 7 Day High	3.696	0.0085

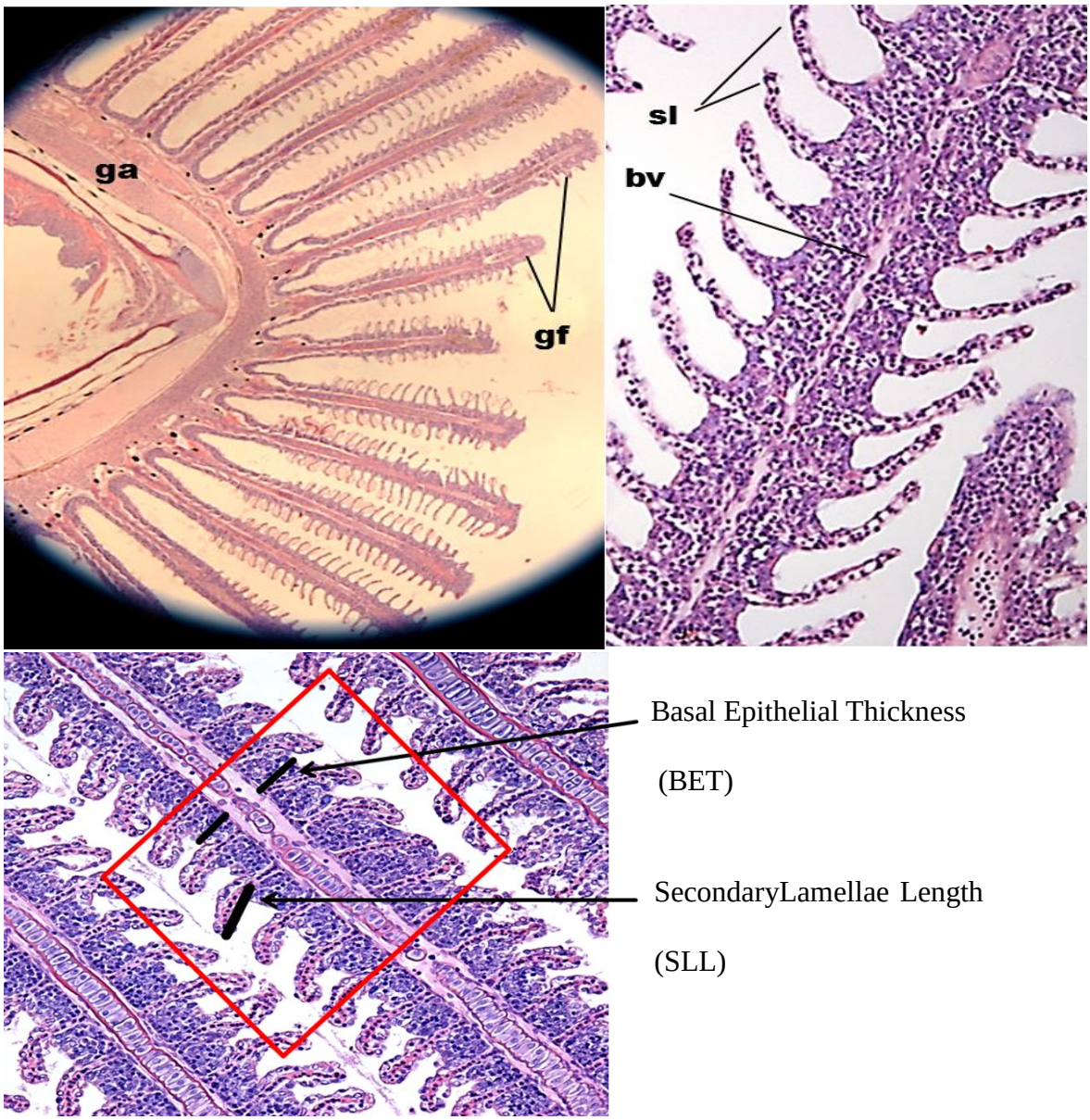

Figure 3.1. Gill anatomy and %PAGE methodology. Stained with H&E. A and B: Gill anatomy A: **ga** gill arch, **gf** gill filament. 4X objective. B: **sl** secondary lamellae, **bv** blood vessel. 40X objective. C: Gill morphometric parameters. 20X objective.

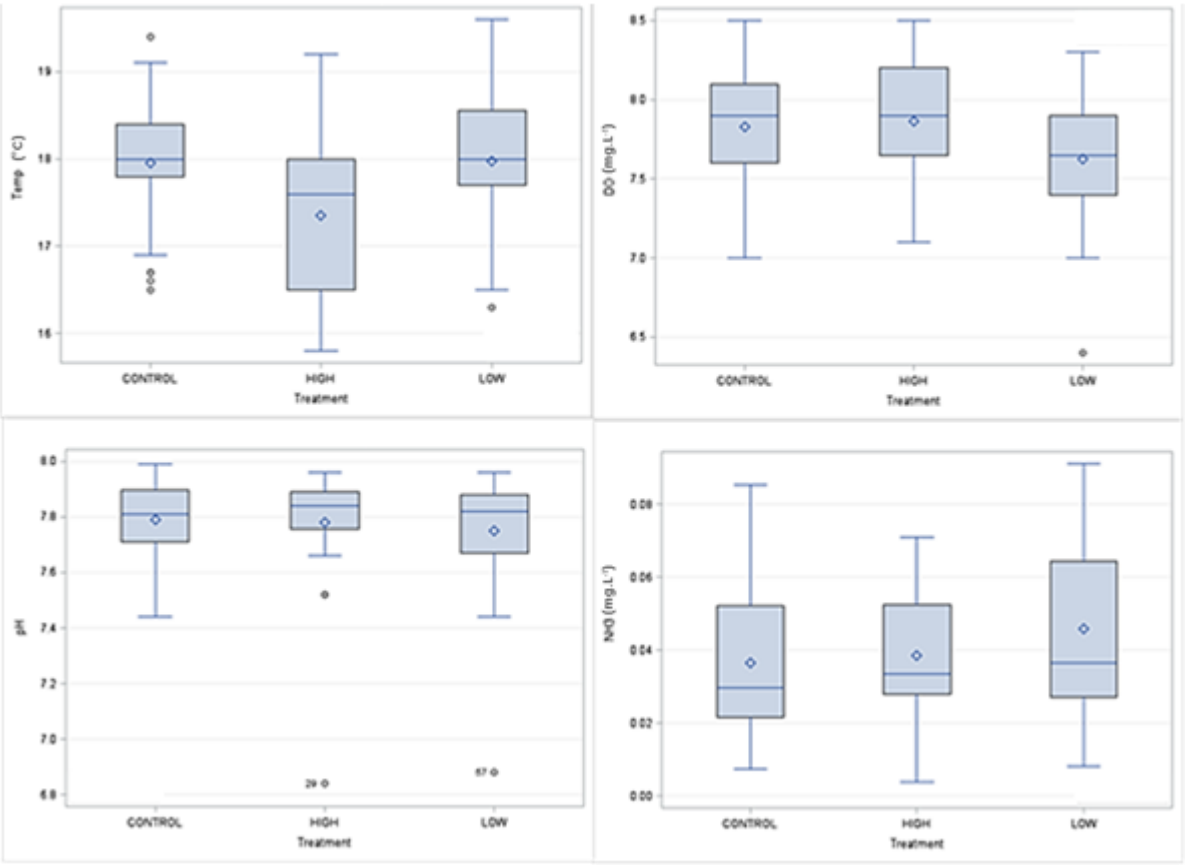

Figure 3.2. Distribution of mean water quality parameters in aquaria for each treatment: Temperature (°C), DO ($mg.L^{-1}$), pH, NH_3 ($mg.L^{-1}$) monitored in each POEA treatment over 7 days. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25^{th} percentile), and upward to the maximum observations (within 1.5 of the 75^{th} percentile). Beyond the upper and lower boundaries, circles indicate outliers.

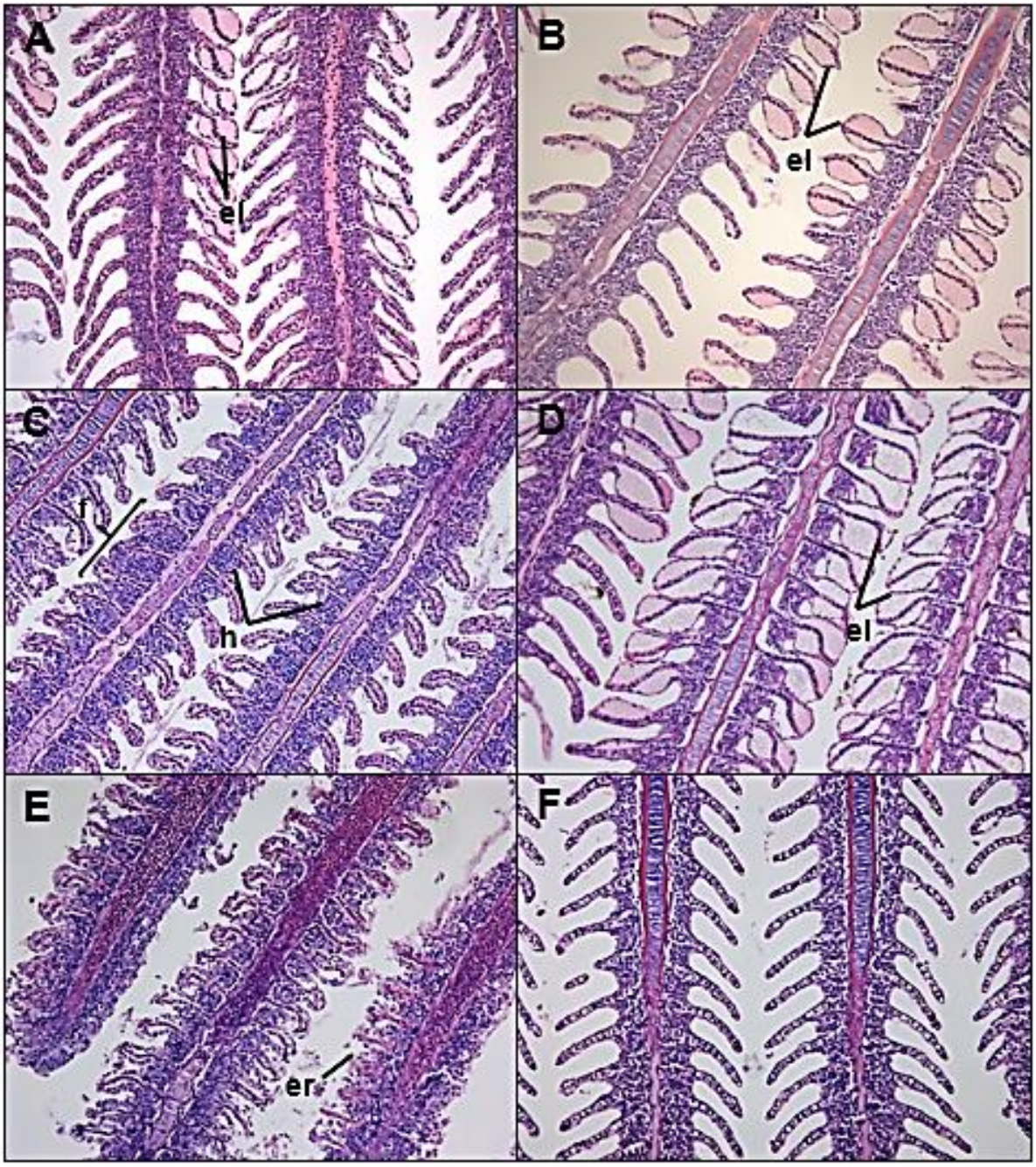

Figure 3.3. Histopathological alterations to Fathead minnow gills. 20X objective. A: Control treatment (no POEA) after 2 day exposure. **el** epithelial lift. B: Control treatment after 7 day exposure. **el** epithelial lift C: Low treatment (10 µg.L^{-1} POEA) after 2 day exposure. **f** fusion of secondary lamellae, **el** epithelial lift, **h** hyperplasia. D: Low treatment after 7 day exposure. **el** epithelial lift E: High treatment (100 µg.L^{-1} POEA) after 2 day exposure. **el** epithelial lift, **h** hyperplasia, **er** epithelial rupture. F: High treatment after 7 day exposure.

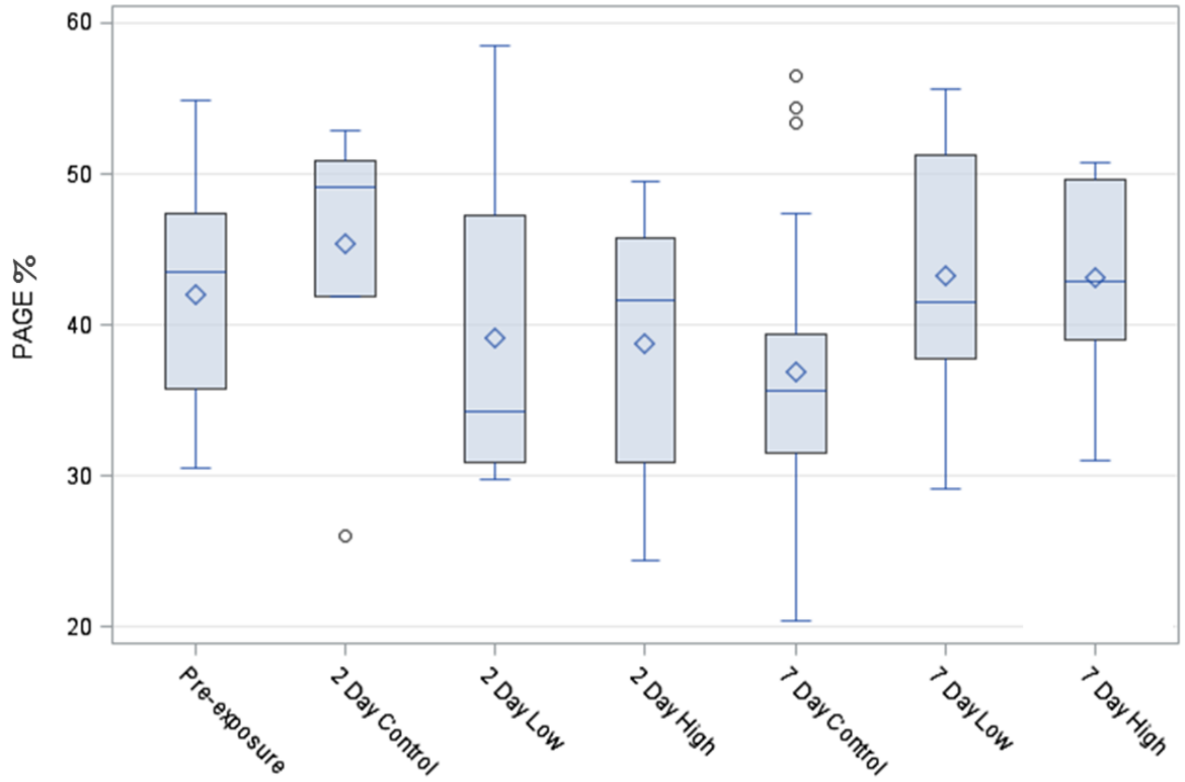

Figure 3.4. Distribution of the PAGE (%) calculated in Fathead minnow gills sampled from all POEA treatments and exposures durations. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25th percentile), and upward to the maximum observations (within 1.5 of the 75th percentile). Beyond the upper and lower boundaries, circles indicate outliers. The two-way ANOVA results revealed no significant difference between the exposure duration, the treatment, nor the interaction of the two ($p = 0.995$, $p = 0.998$, $p = 0.073$, respectively).

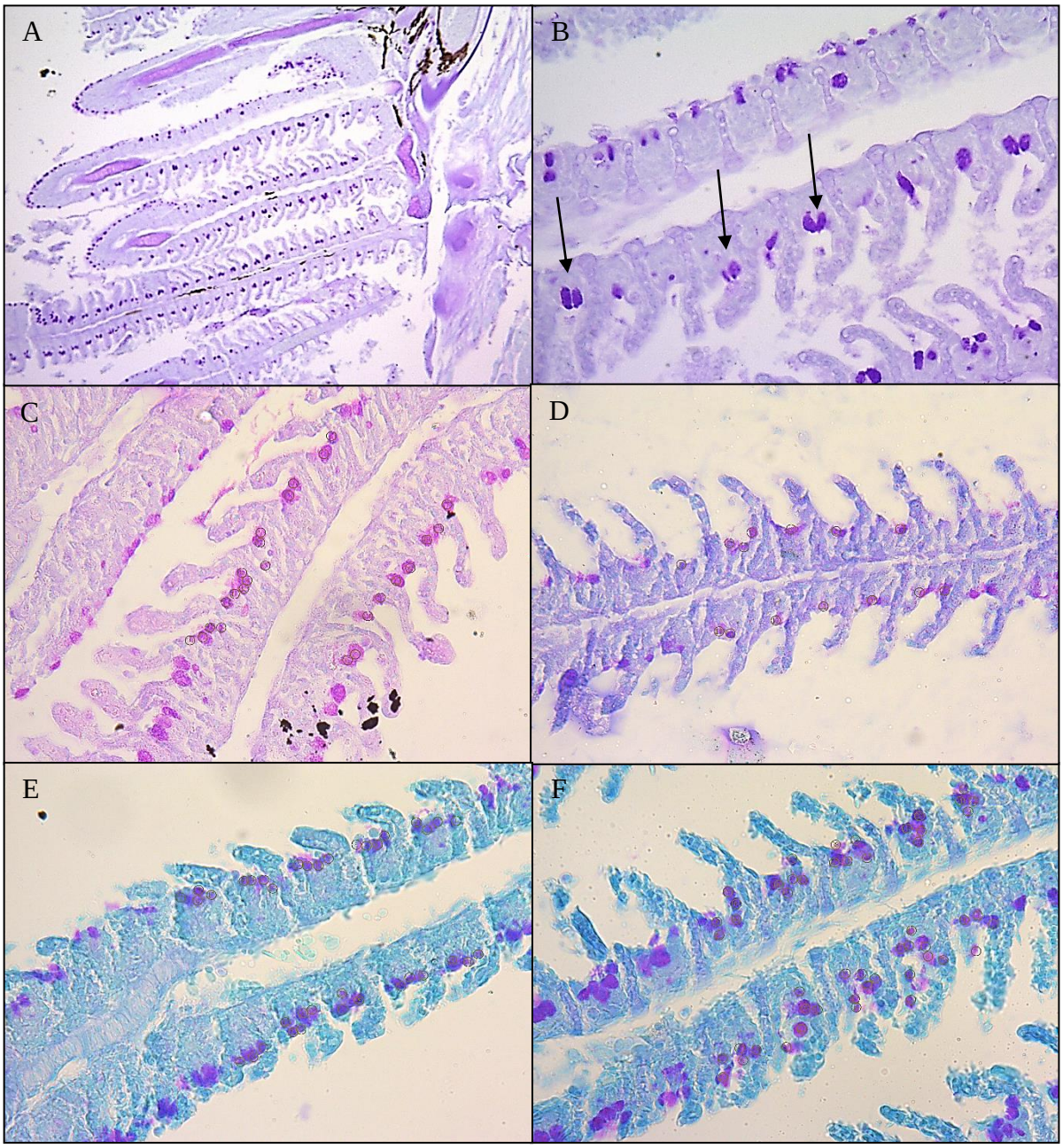

Figure 3.5. PAS stain for mucous cells on Fathead minnow gills. B – F: 40X objective. E and F: with fast green counterstain. A: Control treatment (no POEA) after 7 day exposure. 10X objective. B: Control treatment after 7 day exposure. Mucus cells (arrows) 40X objective. C: Low treatment (10 µg.L^{-1} POEA) after 2 day exposure. D: High treatment (100 µg.L^{-1} POEA) after 2 day exposure. E: Low treatment after 7 day exposure. F: High treatment after 7 day exposure.

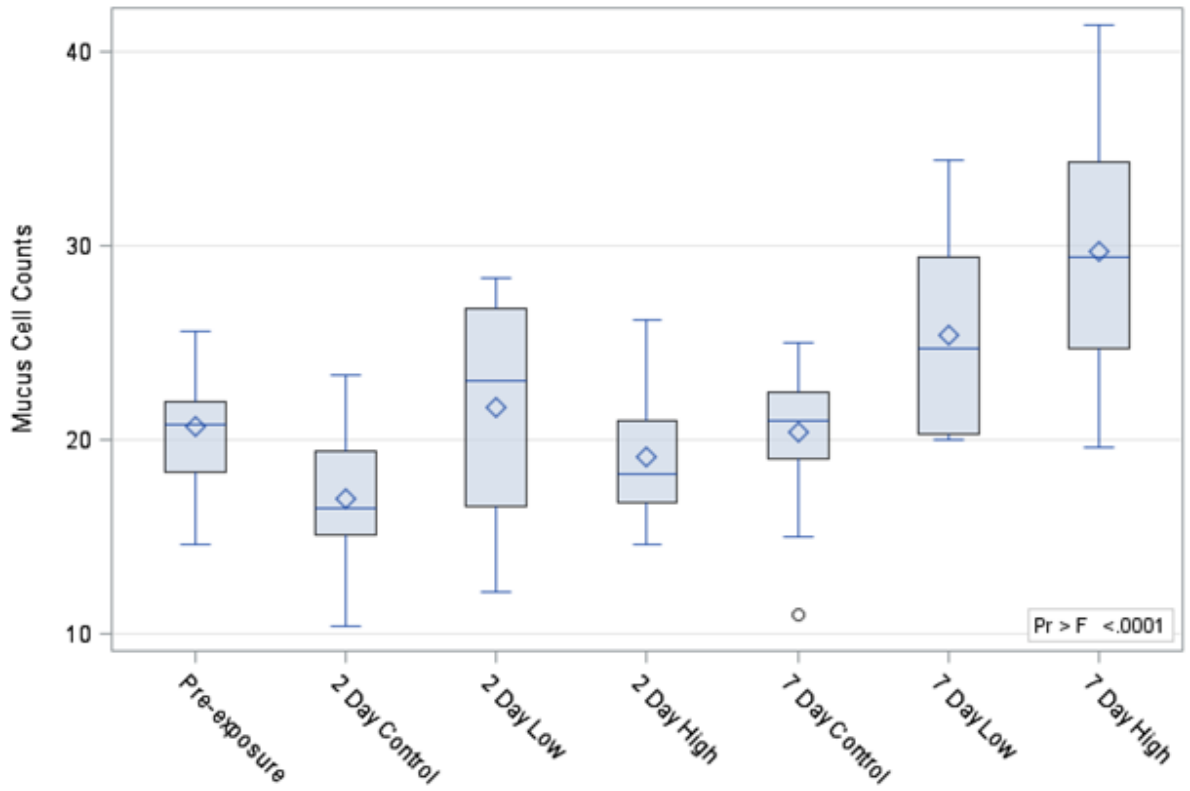

Figure 3.6. Distribution of the mucous cell counts in Fathead minnow gills sampled from all POEA treatments and exposures durations. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25^{th} percentile), and upward to the maximum observations (within 1.5 of the 75^{th} percentile). Beyond the upper and lower boundaries, circles indicate outliers. Two-way ANOVA: significant differences in the exposure duration ($p < 0.001$) and treatment concentrations ($p = 0.005$) of POEA. Bonferroni pairwise comparison post hoc test: 7 Day High significantly greater mucous cell counts than all other treatment groups.

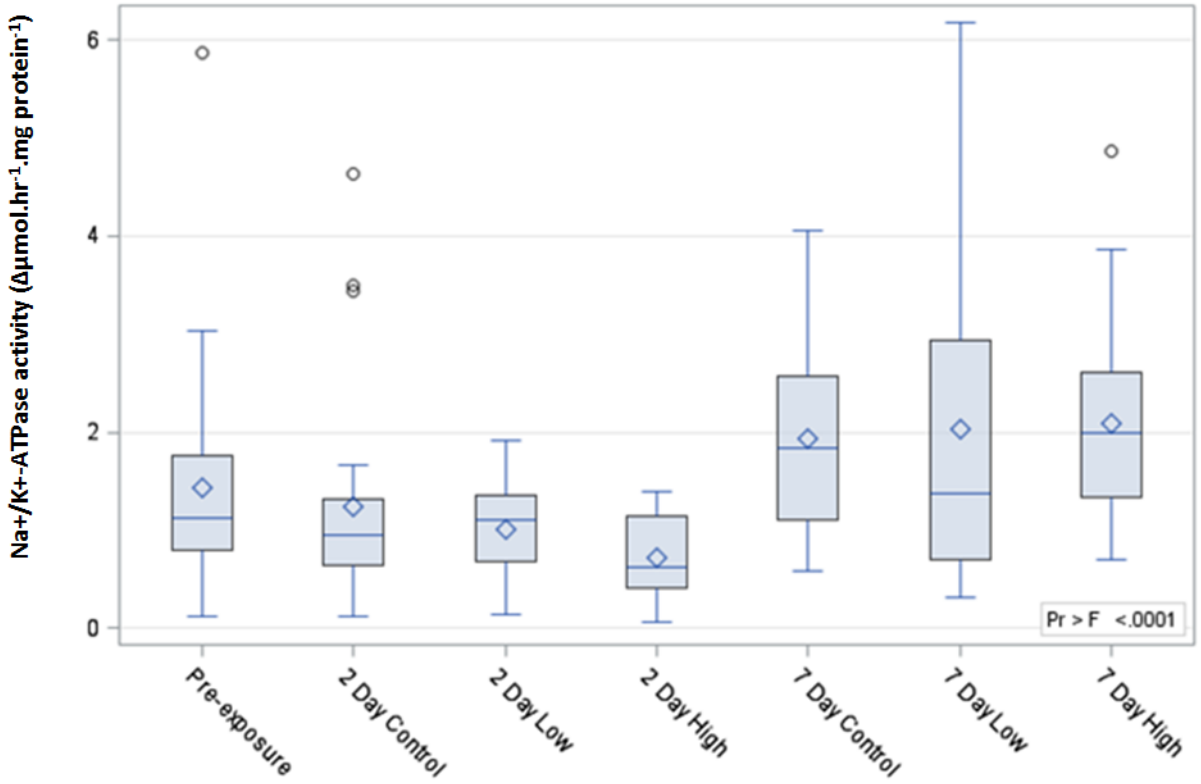

Figure 3.7. Distribution of Na^+/K^+-ATPase activity ($\Delta\mu mol.hr^{-1}.mg\ protein^{-1}$) in Fathead minnow gills sampled from all POEA treatments and exposures durations. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25^{th} percentile), and upward to the maximum observations (within 1.5 of the 75^{th} percentile). Beyond the upper and lower boundaries, circles indicate outliers. Kruskal-Wallis test: significant differences ($p < 0.001$) between the mean Na^+/K^+-ATPase activity in gills of specimens sampled from each of the POEA treatment exposures. Scheffe multiple comparison post hoc test: 2 Day High significantly less Na^+/K^+-ATPase activity in gills than fish sampled from all 7 Day POEA treatments.

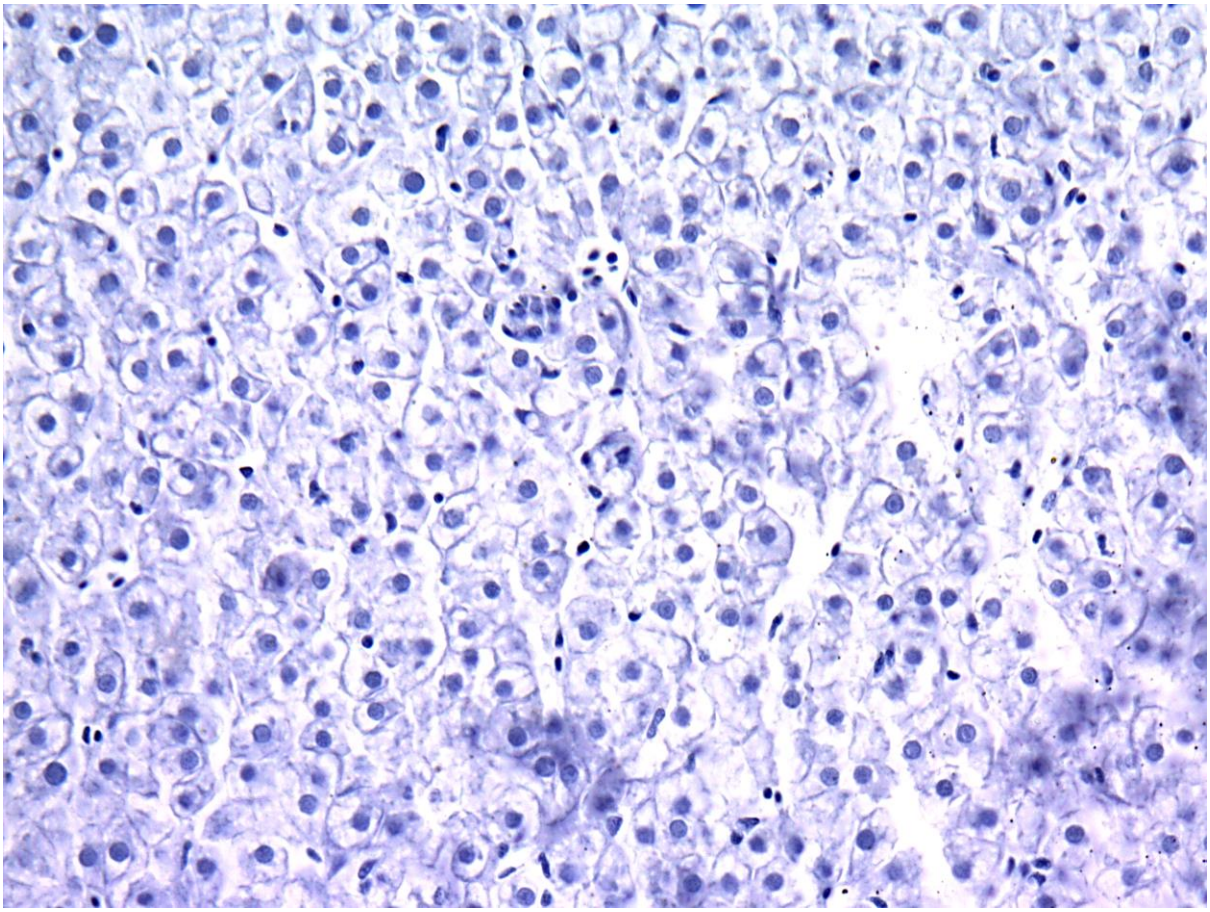

Figure 3.8. Normal histology of Fathead minnow liver sampled from a control tank on the second day of the laboratory POEA exposure experiment (H & E stain, 40X objective mag.).

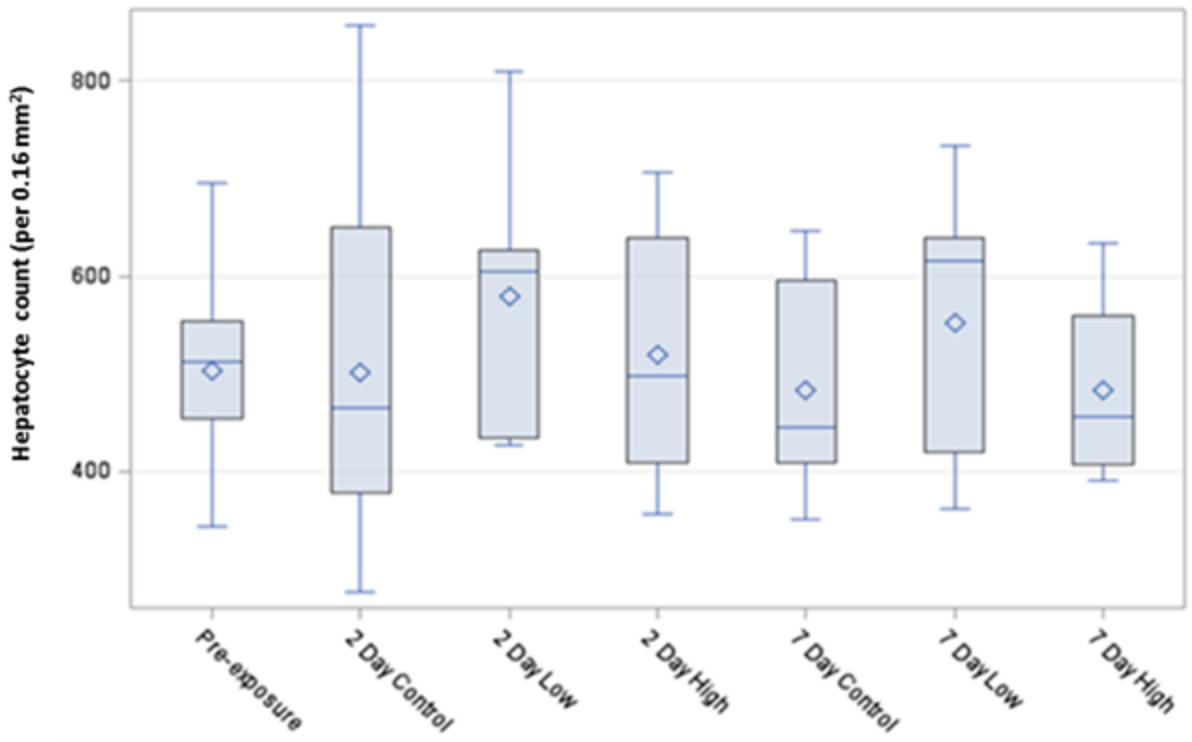

Figure 3.9. Distribution of the hepatocyte counts (per 0.16 mm2 FOV) in Fathead minnow livers sampled from all POEA treatments and exposures durations. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25th percentile), and upward to the maximum observations (within 1.5 of the 75th percentile). Beyond the upper and lower boundaries, circles indicate outliers. Kruskal-Wallis test: no significant difference ($p = 0.743$) between the mean hepatocyte counts in the liver of specimens sampled from each of the POEA treatment exposures.

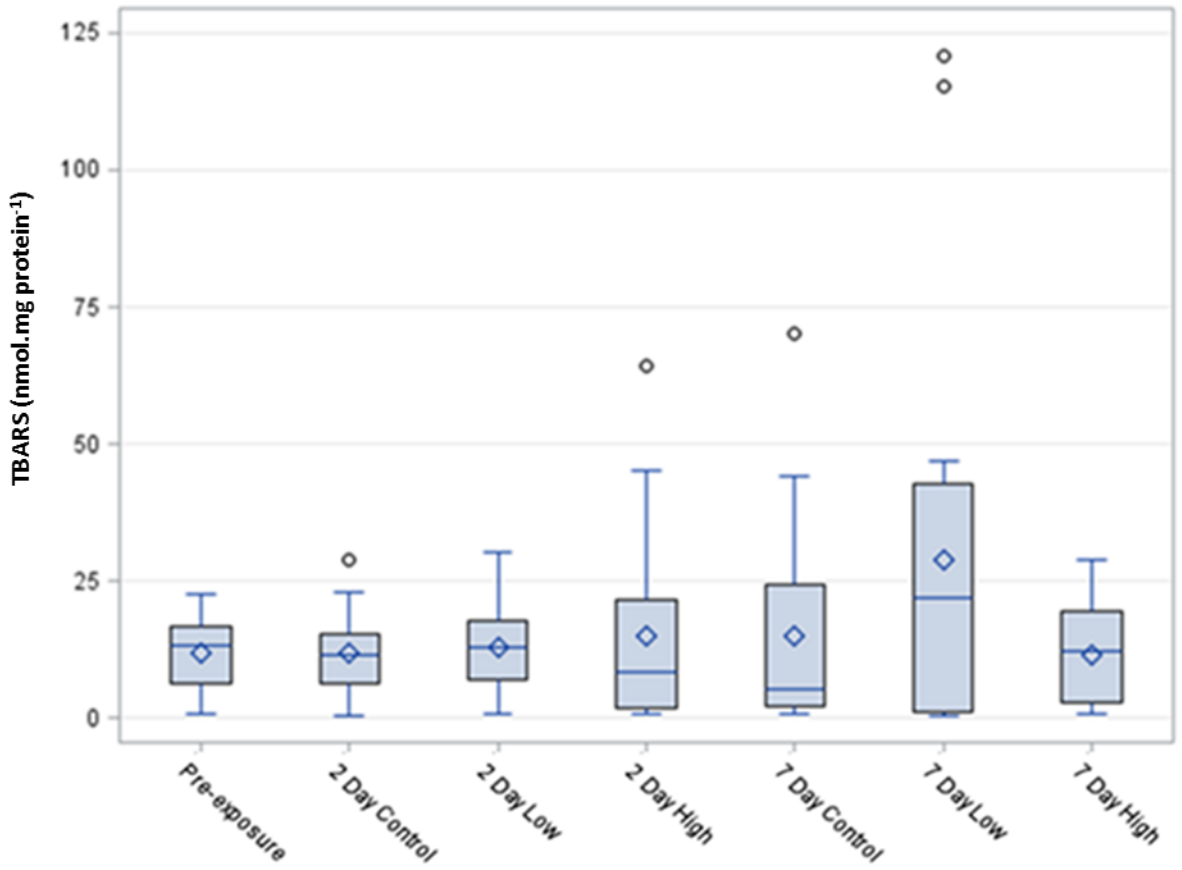

Figure 3.10. Distribution of TBARS (nmol.mg protein-^{1}) produced in Fathead minnow livers sampled from all POEA treatments and exposures durations. The bottom and top edges of the box indicate the intra-quartile range (IQR) (the 25th and 75th percentiles). The diamond marker inside the box denotes the mean value. The line inside the box designates the median value. The whiskers extend downward to the minimum (within 1.5 of the 25th percentile), and upward to the maximum observations (within 1.5 of the 75th percentile). Beyond the upper and lower boundaries, circles indicate outliers. Kruskal-Wallis test: no significant difference ($p = 0.927$) between the mean TBARS levels in the liver of specimens sampled from each of the POEA treatment exposures.

Chapter 4: Risk Assessment for POEA and Final Discussion

4.1. Summary

In Chapter 2, the environmental fate of POEA was investigated. A mesocosm study conducted at the ELA, Ontario, Canada, monitored the dissipation time of environmentally relevant concentrations of POEA (10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$) in the water column as well as in aquatic sediments. POEA bound to suspended particulates was also examined.

In Chapter 3, the effects of POEA on gill and liver histology and biochemical function of Fathead Minnows was examined. Fathead minnows in 10 L aquaria were exposed to the same concentrations of POEA as in the mesocosm study for 2 or 7 days. Histopathology was investigated in the gills (portion available for gas exchange, mucous cell counts) and livers (hepatocyte volume index). Biochemical assays were conducted on gills to determine if inhibition of the ion transport enzyme (Na^+/K^+-ATPase) occurred, and on livers to measure lipid peroxidation due to oxidative stress (TBARS) as a result of POEA exposure.

The following Chapter will assess the environmental risk of POEA, discuss the results of both studies in combination, and address considerations for future studies.

4.2. Introduction

The Herbicide Resistance Action Committee (HRAC) classifies herbicides based on their mode of action; glyphosate is a Class 9 herbicide, meaning it acts by inhibiting enolpyruvylshikimate phosphate synthase (EPSP). Glyphosate is a broad spectrum herbicide and is effective only when applied directly to foliage. To enhance foliar uptake, glyphosate is most often formulated along

with the adjuvant POEA. This enhanced efficacy in controlling nuisance plants tends to increase non-target toxicity. POEA is a non-ionic surfactant frequently added to glyphosate-based herbicides as a wetting agent. It generally constitutes less than 15% of the herbicide formulation by weight (Giesy et al., 2000).

Previous risk assessments have been performed for both glyphosate and whole formulation Roundup®™ in aquatic ecosystems; however, POEA alone has not been assessed (Solomon et al., 2003; Giesy et al., 2000). While risk assessments for the active ingredient, glyphosate, indicate relatively low risk to aquatic systems, other studies which evaluate Roundup® (formulation including 41% glyphosate and 15% POEA) show greater toxicity. Therefore, the investigation of POEA is more relevant with respect to potential for aquatic ecosystem toxicity (Howe et al., 2004).

The 2012 *Canadian Water Quality Guidelines (CWQG) for Glyphosate* by the Canadian Council of Ministers of the Environment (CCME) stated that monitoring for glyphosate alone could underestimate risk to aquatic organisms as a result of the spill of a formulated product containing POEA. To address this issue, CCME is considering developing water quality guidelines for POEA.

In addition, the Pesticide Management Regulatory Agency (PMRA) noted the need for fate and effects data to Fisheries and Oceans Canada (DFO), especially in shallow ecosystems. Shallow, freshwater ecosystems are considered to be at greatest risk because they have a lower ability to dilute contaminants and are also rich in organic matter such as algae, macrophytes, invertebrates, amphibians and juvenile fish and also terrestrial vertebrates such as waterfowl (Solomon et al., 2003) (Figure 4.1).

The following assessment will evaluate the environmental risk of POEA to non-target aquatic organisms in the case of incidental, direct overspray to a shallow freshwater body. In order to determine if a risk is imminent, a probabilistic approach was taken, which utilizes the limited environmental exposure and toxicity data available in the literature in combination with the results of my thesis research.

4.3. Risk Analysis

4.3.1. Exposure characterization

In agriculture, the use of glyphosate-based herbicides in the U.S. has more than doubled since the advent of Roundup Ready® crops in 1996 (Perez et al., 2011). Glyphosate-based herbicides are typically applied at a rate of 1.79 kg (a.e.).ha^{-1} (Giesy et al., 2000). It is assumed that approximately 8% - 15% of the glyphosate formulation by weight is POEA, so at this application rate there is 0.18 - 0.34 kg.ha^{-1} POEA applied with an average of 0.26 kg.ha^{-1}.

In forestry, herbicides are used to manage unwanted trees, brush and other competing vegetation in order to promote and maximize the regrowth of species valuable to the timber industry. Typically, glyphosate-based herbicides are applied between July and September within five years after harvest and each site receives a maximum of two treatments over a period of 50 - 80 years (Thompson et al., 2011). In Ontario, approximately 70,000 ha are treated annually. This accounts for a third of the area harvested per year. The average application rate for Vision® in Ontario is 1.92 kg.ha^{-1} (Thompson et al., 2003).

Since there are no documented studies for POEA monitored in the environment, the use of models is required to estimate environmental exposure concentrations (EEC). In Canada, the

forest pool model estimates pesticide concentrations in water assuming direct overspray. The rate of application is divided by the volume of a hectare at 0.15m depth (100 x 100 x 0.15). As noted above, application rates of glyphosate-based herbicides is approximately 1.79 kg (a.e.).ha^{-1} (Geisy et al., 2000) and in Ontario forestry applications are typically 1.92 $kg.ha^{-1}$ (Thompson et al., 2003). Using the maximum percent of POEA in formulation (15%), we can determine the EEC in agricultural and forest ponds to be approximately 195 $\mu g.L^{-1}$ in Manitoba and 180 $\mu g.L^{-1}$ in Ontario, respectively.

4.3.2. Effects characterization

Based on POEA's short dissipation time in water and rapid binding to suspended particulates and sediment, it is likely that exposures to aquatic organisms will be a result of spray-drift or accidental, non-target overspray rather than run-off or leaching. Due to seasonal applications of herbicides in both agriculture and forestry, exposures to aquatic organisms will be episodic and acute.

Organisms with a single epithelium layer such as frogs, tadpoles, and fish will be at greater risk. The latter two organisms also possess gill structures, which tend to be highly sensitive to surfactants.

Little research has been published on the effects of POEA; however, much work has been done on the effects of glyphosate formulations (Roundup® and Vision®) on aquatic systems and in particular, fish species. Toxicity studies conducted using glyphosate-based herbicides in full formulation have confirmed a greater toxicity than glyphosate alone. This observation is widely attributed to the adjuvant surfactant, POEA (Folmar et al., 1979; Wan et al., 1989; Giesy et al., 2000; Solomon et al., 2003; Thompson et al., 2004; Howe et al., 2004; Reylea, 2005).

A literature review identified studies which reported species sensitivity to POEA, glyphosate, and the formulated product (LC50/EC50 values). The species that were most sensitive (greatest toxicity at lowest exposure) were selected to represent a Tier 1 worst-case scenario risk assessment (Table 4.1 & Table 4.2). Species sensitivity distribution (SSD) curves were constructed to demonstrate the relative sensitivities of aquatic species exposed to POEA (Figure 4.2, Figure 4.3). The SSDs were generated by organizing the toxicity data according to sensitivity and then ranking them using the Weibull equation: [rank / (n+1)]*100 (Zajdlik and Associates, 2005). Percent probability rank was plotted along the y – axis. Reported toxicity concentrations were plotted along the x – axis. SSD curves were constructed in SigmaPlot.

4.3.3. Risk characterization

The potential risk of POEA to non-target organisms was derived using the EC/LC50 values for the most sensitive species of primary producers, zooplankton, fish and amphibians (Table 4.1). The forest pool model provides the maximum level of exposure estimated.

The hazard quotient (HQ) is a conservative method used in Tier 1 risk assessment scenarios (Table 4.3). If the HQ is less than 1, then it can be safely presumed that there is no risk of effects on non-target organisms. If the HQ is greater than 1, a Tier 2 risk assessment scenario (semi-quantitative; conservative but more realistic estimates of exposure and effects) should be considered. The following equation was used to calculate the HQs for POEA of species representative of each trophic level based on uses in Manitoba agriculture and Ontario forestry:

$$HQ = \left(\frac{EEC}{TOX}\right) xUF$$

Where EEC is environmental exposure concentration ($mg.L^{-1}$), TOX is species effects concentration ($mg.L^{-1}$), and UF is uncertainty factor.

An uncertainty factor of 1000 was applied based on guidelines for Environmental Assessment of Priority Substances under the Canadian Environmental Protection Act (Environment Canada 1997)

The hazard quotients expressed in Table 4.3 imply a risk to species in all trophic levels for POEA uses in agriculture and forestry. This could have a profound effect on aquatic ecosystems at a population and community level. Therefore, a higher Tier analysis would be recommended.

4.4. Final Discussion and Uncertainty

4.4.1. POEA fate and toxicity in the water column

Until the 2012 ELA POEA mesocosm study (Chapter 2), no environmental fate data of POEA were available. The assumption that POEA does not leach from soils or enter aquatic systems via runoff as noted in the above USEPA model and in the risk assessment of Giesy et al. (2000) were model derived conclusions that were necessitated by the lack of fate data for POEA in natural settings. The findings of this study confirmed that POEA rapidly dissipates from the water column. However, dissipation times were determined to be considerably less than those modeled by Giesy et al. (21 – 42 days). In fact, POEA was no longer detectable in the water column of enclosures after 9 days, and dissipation times were quite similar to those determined by Wang et al. (2005) in a laboratory study (DT50 = 13 h with 3% TOC, and 18 h with 1.5% TOC). Conversely, the results of the mesocosm study did not identify differences in the dissipation time of POEA in the water column between enclosures that were open-to or closed-off from the

underlying sediment, nor were correlations found between TOC concentration and POEA dissipation in each mesocosm. The dissipation of POEA in the water column was a key element of interest in the joint ELA and laboratory study. Wang et al. (2005) reported that POEA in water-only aquaria maintained relatively the same concentration over 96 hrs. Unfortunately, in the laboratory study, POEA concentrations were inconclusive due to analytical uncertainty. Therefore, comparisons of POEA DT50 in the laboratory study and field study were not possible. This is not to say that POEA does not bind to particulates, but simply that TOC did not appear to influence the dissipation time of POEA in the present study.

In both the ELA and laboratory study, fish were exposed to 10 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$ of POEA. In the ELA study, the analysis of POEA concentration in fish tissue was originally intended; unfortunately, due to funding limitations, only Fulton's condition factor was assessed. After one week, Fathead minnows in two out of the three low exposure mesocosms were in notably poorer condition that those from the surrounding lake. In contrast, FCF of Fathead minnows in the laboratory exposure only revealed significant differences between length of exposure (2 and 7 days), but not treatments.

Exposure duration was also revealed to be an influential factor in histopathological alterations and biochemical function. Fish exposed to 100 $\mu g.L^{-1}$ of POEA for 2 days demonstrated inhibition of Na^+/K^+-ATPase activity in gills as compared to fish exposed for 7 days. Similar results were expected from the PAGE percentage given that gill tissue should reflect immediate response to POEA and then recover as POEA dissipated from the water column. However, no significant differences in PAGE between treatment and controls were observed. On the contrary, greater mucous cell counts in gills were observed in all treatments after the seventh day of POEA exposure,

Temperature, DO, pH, and NH_4^+ did not exceed levels considered harmful to Fathead minnows (Klinger et al., 1982; Duffy 1998). According to the US EPA, NH_3 levels exceeding 0.02 $mg.L^{-1}$ (Willingham, 1976) may cause adverse effects. Unionized ammonia levels greater than 0.02 $mg.L^{-1}$ in all tanks may have led to observed effects despite the fact that previous studies have demonstrated that Fathead minnows have a robust tolerance (Reinbold et al. 1982; Thurston et al. 1986).

Water quality parameters as well as nutrients were also monitored in the ELA fate experiment. Although NH_3, and NH_4^+ were not individually measured, total dissolved nitrogen (TDN) was. Since mucous cell counts and protein assays were not performed on Fathead minnows exposed to POEA in the enclosures, we can only look to FCF to compare if nitrogen appeared to have an effect on condition factor. Elevated levels of nitrogen were observed in three of the enclosures (Control B, Low B and High A) immediately following treatment (July 25, 2012). The FCF determined for Fathead minnows in these enclosures after one week did not reflect any decreases in condition. In fact, FCF was greatest in Control B and High A as compared to fish sampled from all other enclosures. As stated, only TDN was monitored, and therefore it cannot be said with certainty whether unionized ammonia levels approached the threshold deemed safe by the US EPA of 0.02 $mg.L^{-1}$ (Willingham, 1976) in the mesocosms during the ELA POEA exposure.

The standard deviation reported in all histopathological analyses and the TBARS assay was quite high, emphasizing the high variability within the small sample sizes. The TBARS assay was originally intended to be paired with a glutathione assay; however, small sample volumes of liver did not allow for this supportive analysis to be conducted. Subsequent evaluation of glutathione ratios would have provided reassuring evidence as to whether POEA induced oxidative stress in the liver of exposed fish.

No fish mortalities occurred during the POEA laboratory exposure, and those observed during the ELA mesocosm experiment were likely not related to POEA exposure as fish mortality was equally observed in the control enclosures.

4.4.2. POEA bound to suspended particulates

An important aspect relating to the fate of POEA in the environment that was researched during the ELA experiment, but was not investigated in the laboratory exposure, was POEA's affinity to suspended particulates and underlying aquatic sediments. Howe et al. (2004) noted that because POEA adheres to particles that organisms may feed on, exposure may be higher than expected when only waterborne exposures are considered. The fate of POEA in sediments and toxicity to benthic organisms is poorly understood. The ELA POEA mesocosm experiment was designed to fill these data gaps, which were identified as a critical need for the Ecological Risk Assessment process by Health Canada's Pest Management Regulatory Agency (PMRA).

Analysis of particulates filtered from water samples, revealed that only a fraction of the initial POEA concentration was bound to suspended particulates. The maximum being 19.6 $mg.g^{-1}$ POEA (DT50 = 14.7 h) sampled from the high treatment, closed-bottom mesocosm (High C) at 1 hour following the initial dose. Furthermore, no POEA was detectable after 72 hrs in most of the treatment enclosures. It is likely that particulates bound with POEA settled out of the water column after 72 hrs.

The lack of desorption and short presence of POEA bound to particulates suggests that aquatic organisms would not have the opportunity to ingest toxic levels of POEA while in the water column. In addition, a review of the bioaccumulation potential of surfactants concluded that there is no evidence to support concerns with respect to biomagnification nor long-term retention

of bioaccumulated surfactants (McWilliams et al., 2002).

4.4.3. POEA in aquatic sediments

What is of greatest concern, is the prolonged presence of POEA in aquatic sediments. Howe et al., (2004) noted that environmental levels and persistence of POEA after field applications is lacking but that these data are required for a complete assessment of the acute and chronic toxicity of glyphosate formulations. The final samples analyzed from October 8, 77 days after initial dosing, still had detectable levels of POEA in both high treatment mesocosms (156.6 and 389.9 $ng.g^{-1}$). The DT50 of mean POEA in the sediments of high treatment corrals was 42.5 days. These findings lend support to the concern that benthic organisms are at an elevated risk from POEA exposure.

4.4.4. Risk assessment for POEA in aquatic ecosystems

In a true agricultural setting, glyphosate-based herbicides may be applied multiple times throughout the season: (1) Pre-seed burn-off of weeds in the spring, (2) up to two applications for pre-harvest weed control, (3) harvest desiccation, and (4) a post-harvest burn-off of weeds in the fall. The one-time environmentally relevant dose of POEA investigated in both the ELA mesocosm fate study and the laboratory biological effects experiment may not be a realistic representation of the true environmental circumstances.

Due to the limited exposure and toxicity data available for POEA, a probabilistic risk assessment was performed. The obvious factor when considering uncertainty is the extrapolation from mathematical and predictive models to a real world context. It is difficult to say without direct application how accurate a SSD curve is at estimating actual toxicity values in various species. In

this risk analysis, very limited data was available for POEA toxicities; whereas in other risk assessments the range and density of species exposed to glyphosate and its subsequent formulation was much more robust.

In addition, toxicity of the stressor may increase or decrease when in the presence of other compounds. In this case, Figure 4.3 shows the toxicity of POEA to be greatest on its own than in combination with glyphosate. However, it is unlikely that POEA would enter the environment unaccompanied by the active ingredient, glyphosate.

4.5. Recommendations

4.5.1. Environmental fate of POEA

To further investigate the fate of POEA in aquatic ecosystems, a multiple site study that considers various lake characteristics (such as rocky bottom and low TSS) should be conducted to effectively assess the affinity of POEA to suspended particulates or high organic content in natural systems. Multiple doses of POEA to aquatic environments should also be investigated. As should the degradation of POEA in aquatic sediments over winter, following multiple doses, to assess for season-to-season accumulation. The actual composition of sediments should also be noted.

Another complication in the ELA study was the lack of replicate treatments. Mesocosms with open-bottom designs had POEA treatments in duplicate, with no replications of treatments in closed-bottom designs. It's strongly suggested that any further studies investigating the influence of aquatic sediments on the dissipation time of POEA from the water column be run in at least triplicate.

Further research should be conducted in a field setting to monitor environmental exposure concentrations of POEA in aquatic systems in proximity to agriculture and forestry areas to which glyphosate formulations are applied, as this information is lacking in the primary literature and its results would provide a more definitive scope on the susceptibility of aquatic organisms to POEA as a whole.

4.5.2. POEA effects on normal histology and biochemical parameters

To mimic multiple doses of POEA in the environment, a laboratory exposure should be conducted in a flow-through system with POEA concentrations maintained at environmentally relevant concentrations for 6 months (an entire growing season).

With respect to the histopathological effects investigated in the gills of Fathead minnows exposed to POEA, the specific glycoproteins produced from the mucous cells could be examined. Periodic acid-Schiff is generally used for neutral glycoproteins (McManaus 1948), but histochemistry techniques such as Alcian blue (AB) at pH 1.0 to highlight sulphated glycoproteins (Lev et al., 1964) and at pH 2.5 for acid glycoproteins (Mowry 1956) could also be employed to characterize the exposure response to POEA (Yamabayashi, 1987; Moron et al., 2009). It's likely that neutral glycoproteins would dominate, as they are thought to protect and lubricate gill epithelium against physical injuries (Moron et al., 2009). However, the acidic and sulphated acid glycoproteins may also be present if the fish is in a weakened state, as these mucosubstances are thought to prevent the propagation of harmful microorganisms (Mittal et al., 1994).

Many studies pair chloride cells with the analysis of mucous cells in gills (Moron et al., 2009; Pereira et al., 2012). Both cell types are known to proliferate under stressful conditions and as a

result, increase the diffusion distance of respiratory gases (Fernandes et al., 1998). Chloride cells are also considered to be the major site of Na^+/K+-ATPase activity and have shown a positive correlation between Na^+/K^+-ATPase activity and chloride cell density (Dang et al., 2000). The proliferation in chloride cells in the gills of fish exposed to pollutants is considered a compensatory mechanism against the inhibition of Na^+/K^+-ATPase (Dang et al. 2000). Aside from the Na^+/K^+-ATPase assay conducted in the present study, fluorescent labelling of ouabain-sensitive Na^+/K^+-ATPase for microscopy analysis as described by McCormick et al., (1990) could provide more confidence in the results than just the assay alone.

Cortisol levels could also be investigated with respect to Na^+/K^+-ATPase expression, as increasing cortisol levels stimulate restoration of enzyme driven ion regulation in chloride cells (Balm et all., 1987; Dang, 2000).

The primary complications experienced during histopathological analysis of livers, was locating a field-of-view of the section that was suitable for microscopy analysis. Working with exceptionally small volumes of tissue can be difficult in paraffin embedding, prolonged dehydration may cause shrinkage and hardening of tissue leading to compression and tearing while sectioning, as evidenced in the present study. It is recommended that future analysis of small tissue, instead be processed for plastic embedding and stained with toluidine blue. This will guarantee improved tissue structure maintenance and a higher resolution.

Biochemical analysis of livers also proved challenging. TBARS values showed a large degree of variation. It is recommended that future studies pair the TBARS assay with a test for glutathione (oxidized/reduced) to confirm levels of oxidative stress.

4.6. Conclusion

Based on the results of the ELA POEA environmental fate study and the laboratory POEA exposure study, with further consideration of the hazard quotient method using estimated exposure concentrations and the most sensitive species toxicities, the risk to aquatic organisms at all trophic levels with the incidental application of POEA to agriculture and forest ponds would be minimal. However, both direct and indirect effects may be observed resulting in adverse effects at the population and community levels in aquatic ecosystems. A Tier 2 risk assessment is recommended.

Table 4.1. Acute toxicity of POEA to amphibians, fish, aquatic invertebrates and aquatic plants

Common Name	Species	Effect Measure	End Point	Toxicity ($mg.L^{-1}$)	Source
AMPHIBIANS					
Green frog	*Rana clamitans*	96hr LC50	Mortality	1.1	Howe et al. 2004
Africa clawed frog, embryos	*Xenopus laevis*	96hr LC50	Mortality	6.8	Perkins et al. 2000
FISH					
Rainbow trout	*Oncorhynchus mykiss*	96hr LC50	Mortality	0.68	Mayer et al. 1986
Fathead minnow	*Pimephales promelas*	96hr LC50	Mortality	1	Folmar et al. 1979
Bluegill sunfish	*Lepomis macrochirus*	96hr LC50	Mortality	1	Mayer et al. 1986
Chum salmon	*Oncorhynchus keta*	96hr LC50	Mortality	2.4	Wan et al. 1989
Sockeye salmon, fry	*Oncorhynchus nerka*	96hr LC50	Mortality	2.6	Servizi et al. 1987
Chinook salmon	*Oncorhynchus tshawytsha*	96hr LC50	Mortality	2.8	Wan et al. 1989
Pink salmon	*Oncorhynchus gorbuscha*	96hr LC50	Mortality	2.8	Wan et al. 1989
Channel catfish	*Ictalurus punctatus*	96hr LC50	Mortality	3	EPA App J 2008
Coho salmon	*Oncorhynchus kisutch*	96hr LC50	Mortality	3.2	Wan et al. 1989
INVERTEBRATES					
Freshwater mussel, glochidia	*Lampsilis siliquiodea*	48hr EC50	Shell Closure	0.5	Bringolf et al. 2007
Freshwater mussel, juvenile	*Lampsilis siliquiodea,*	96hr EC50	Foot Movement	3.5	Bringolf et al. 2007

Common Name	Species	Effect Measure	End Point	Toxicity ($mg.L^{-1}$)	Source
Marine copepod	*Acartia tonsa*	48hr LC50	Mortality	0.57	Tsui & Chu 2003
Cladoceran	*Ceriodaphnia dubia*	48hr LC50	Mortality	1.15	Tsui & Chu 2003
Cladoceran	*Daphnia magna*	48hr LC50	Mortality	2	ABC Inc. 1980
Cladoceran	*Daphnia pulex*	96hr EC50		2	Servizi et al. 1987
Midge larvae	*Chironomus plumosus*	48hr EC50	Immobilized	13	Folmar et al. 1979
AQUATIC PLANTS					
Marine diatom	*Skeletonema costatum*	96hr EC50	Growth Inhibition	2.24	Tsui & Chu 2003
Algae	*Pseudokirchneriella subcapitata*	96hr EC50	Growth Inhibition	2.63	Tsui & Chu 2003
MICROBES					
Freshwater/marine bacterium	*Vibrio fischeri*	15min EC50	Luminescence emission	10.2	Tsui & Chu 2003
Freshwater ciliate protozoa	*Tetrahymena pyriformis*	40hr EC50	Luminescence emission	4.96	Tsui & Chu 2003
Marine ciliate protozoa	*Euplotes vannus*	48hr EC50	Luminescence emission	5	Tsui & Chu 2003

Table 4.2. Acute toxicity of POEA, Roundup®, and Glyphosate to fish

			Toxicity ($mg.L^{-1}$)			
Common Name	Species	Effect Measure	POEA[1]	Roundup®[2]	Glyphosate[3]	Source
Rainbow trout	*Oncorhynchus mykiss*	96hr LC50	0.68	8.3	22	[1]Mayer et al. 1986; [2]Folmar et al. 1979; [3]Wan et al. 1989
Fathead minnow	*Pimephales promelas*	96hr LC50	1	2.3	97	Folmar et al. 1979
Bluegill sunfish	*Lepomis macrochirus*	96hr LC50	1	5	140	Folmar et al. 1979
Chum salmon	*Oncorhynchus keta*	96hr LC50	2.4	19	22	Wan et al. 1989
Chinook salmon	*Oncorhynchus tshawytscha*	96hr LC50	2.8	9.6	30	[1, 3]Wan et al. 1989; [2]Folmar et al, 1979
Pink salmon	*Oncorhyncus gorbuscha*	96hr LC50	2.8	31	23	Wan et al. 1989
Channel catfish	*Ictalurus punctatus*	96hr LC50	3	13	130	[1]EPA App J; [2]Mitchell et al. 1987; [3]Folmar et al. 1979
Coho salmon	*Oncorhynchus kisutch*	96hr LC50	3.2	11	36	[1, 3]Wan et al. 1989; [2]Mitchell et al. 1987

Table 4.3. Hazard quotients for POEA uses in Manitoba agriculture and Ontario forestry

Species	Effect Measure	Toxicity ($mg.L^{-1}$)	Ag EEC ($mg.L^{-1}$)	Forestry EEC ($mg.L^{-1}$)	Uncertainty Factor	Ag HQ	Forestry HQ
Green Frog (*R. clamitans*)	96hr LC50	1.1	0.195	0.180	1000	177	163
Rainbow trout (*Oncorhynchus mykiss)*	96hr LC50	0.68	0.195	0.180	1000	287	265
Freshwater mussel (*Lampsilis siliquiodea, glochidia)*	48hr EC50	0.5	0.195	0.180	1000	390	360
Marine diatom (*Skeletonema costatum)*	96hr EC50	2.24	0.195	0.180	1000	87	80
Freshwater ciliate protozoa (*Tetrahymena pyriformis)*	40hr EC50	4.96	0.195	0.180	1000	39	36

See Table 4.1 for references for toxicity values. EEC concentrations calculated in Section 4.2.1.

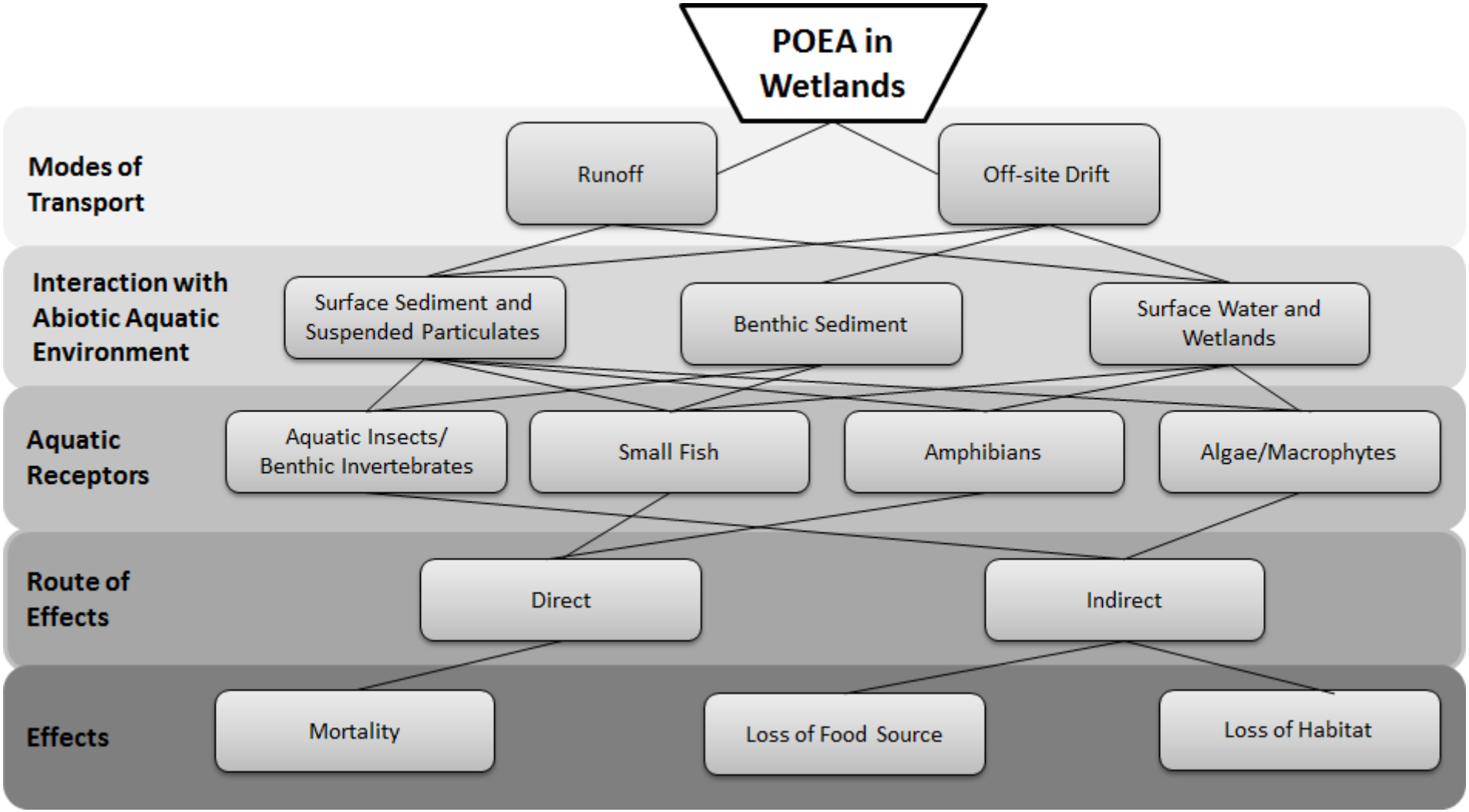

Figure 4.1. Conceptual model illustrates pathways of POEA from source to effects.

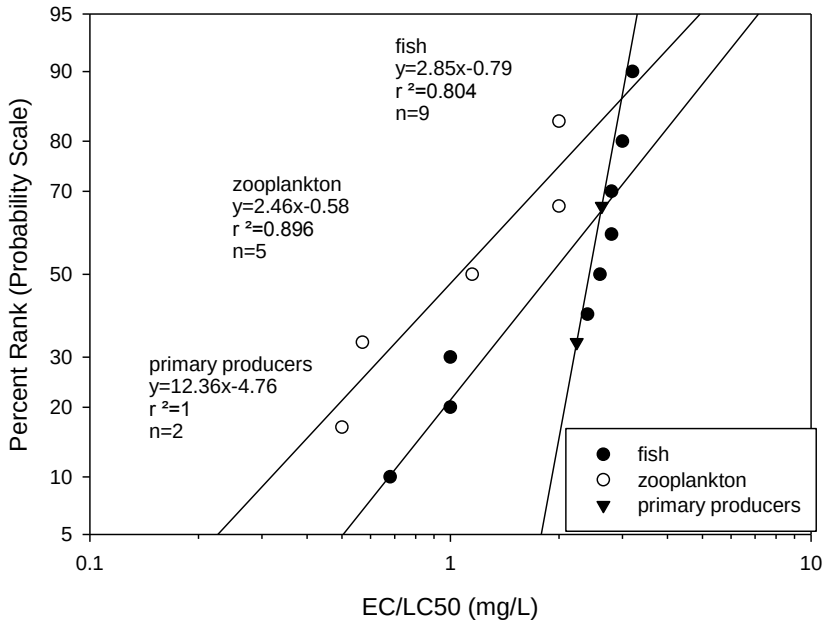

Figure 4.2. Species sensitivity distribution for MON 0818 (POEA) on fish using 96hr LC50s, zooplankton using 48hr EC/LC50s and 96hr LC50s, and primary producers using 96hr EC50s (Table 4.1).

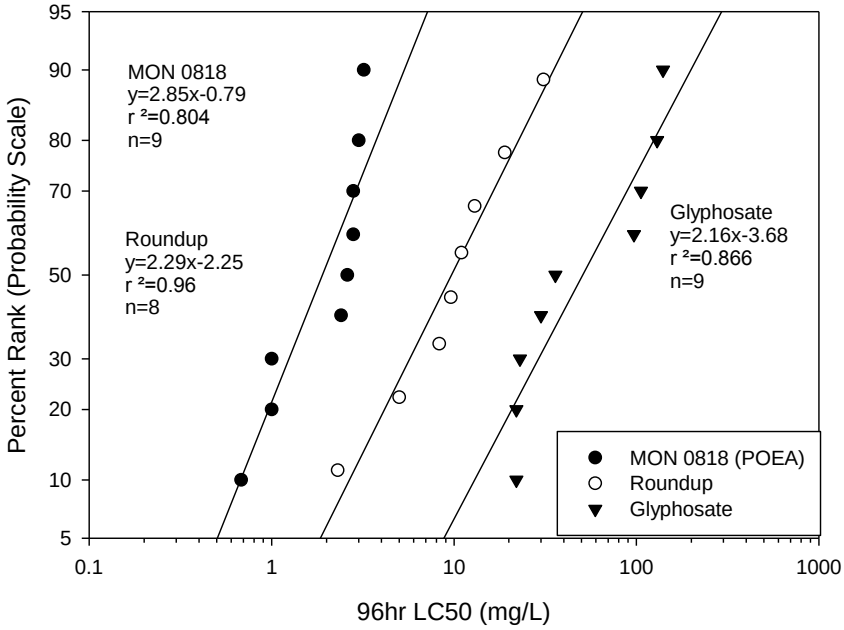

Figure 4.3. Species sensitivity distribution for MON 0818 (POEA), Roundup®™ and Glyphosate on fish using 96hr LC50 ($mg.L^{-1}$). POEA exhibits greatest toxicity to fish. (Table 4.2).

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Appendices

Appendix A: ELA Meteorological Data

Table A.1. Meteorological data for Lake 114 from July 22 – October 10, 2012. ND = No data.

Exp. Day	Date	Max Air Temp. (°C)	Min Air Temp. (°C)	Mean Air Temp. (°C)	Mean Water Temp. (°C)	Total Precip. (mm)	Max Wind Velocity (km/h)	Wind Direction (°)
	Jul-22	30.0	16.0	23.0	25.7	0.0	15.0	228.3
	Jul-23	28.0	18.5	23.3	25.7	0.0	15.6	21.7
1	Jul-24	28.5	15.0	21.8	25.8	0.4	9.1	212.7
2	Jul-25	29.0	17.0	23.0	26.5	25.5	7.5	67.0
3	Jul-26	20.5	15.0	17.8	24.6	4.8	18.5	52.6
4	Jul-27	26.0	12.0	19.0	24.0	0.0	9.4	243.7
5	Jul-28	27.5	14.5	21.0	24.5	0.0	12.6	210.3
6	Jul-29	31.0	18.0	24.5	25.2	11.4	15.4	207.8
7	Jul-30	25.0	16.5	20.8	25.2	0.0	13.7	16.8
8	Jul-31	29.0	15.0	22.0	25.2	0.0	14.5	232.4
9	Aug-01	27.0	20.0	23.5	25.3	2.4	13.2	317.3
10	Aug-02	24.0	15.5	19.8	25.3	2.7	9.2	10.9
11	Aug-03	26.0	15.5	20.8	25.4	8.2	13.7	159.9
12	Aug-04	22.0	14.0	18.0	24.3	1.6	18.0	234.0
13	Aug-05	23.5	10.5	17.0	22.9	0.0	13.9	245.4
14	Aug-06	27.0	16.5	21.8	23.0	0.0	14.5	300.6
15	Aug-07	24.0	13.0	18.5	23.1	0.0	8.7	6.0
16	Aug-08	26.5	13.5	20.0	23.7	0.0	20.6	359.5
17	Aug-09	22.5	14.0	18.3	22.6	0.0	19.9	75.7
18	Aug-10	25.5	11.0	18.3	22.8	0.0	7.9	208.2
19	Aug-11	26.5	12.5	19.5	23.1	0.0	9.3	182.8
20	Aug-12	24.5	14.0	19.3	22.9	6.1	9.8	282.3
21	Aug-13	23.0	11.5	17.3	22.7	0.0	10.2	277.2
22	Aug-14	24.0	12.0	18.0	22.9	0.0	6.9	312.4
23	Aug-15	20.5	13.0	16.8	22.1	19.5	16.0	183.6
24	Aug-16	17.0	10.5	13.8	20.4	0.0	16.4	329.6
25	Aug-17	21.5	7.5	14.5	19.7	0.0	11.2	261.3
26	Aug-18	20.5	11.0	15.8	20.0	0.0	12.1	14.6
27	Aug-19	21.5	6.5	14.0	20.2	0.0	8.7	321.5
28	Aug-20	25.5	12.5	19.0	20.6	0.0	10.2	308.0
29	Aug-21	27.5	12.5	20.0	20.8	0.0	18.3	217.6
30	Aug-22	29.0	14.0	21.5	21.8	0.0	10.5	61.9
31	Aug-23	29.0	16.0	22.5	22.6	2.1	13.0	135.2

Exp. Day	Date	Max Air Temp. (°C)	Min Air Temp. (°C)	Mean Air Temp. (°C)	Mean Water Temp. (°C)	Total Precip. (mm)	Max Wind Velocity (km/h)	Wind Direction (°)
32	Aug-24	25.5	15.0	20.3	22.9	7.6	7.4	211.3
33	Aug-25	23.0	17.0	20.0	22.4	0.1	15.1	226.2
34	Aug-26	22.5	14.5	18.5	21.2	0.0	17.2	246.8
35	Aug-27	23.0	14.0	18.5	21.1	0.0	9.9	345.6
36	Aug-28	26.0	12.0	19.0	21.6	0.0	6.8	246.6
37	Aug-29	32.0	15.5	23.8	22.0	0.0	15.2	196.2
38	Aug-30	27.0	19.0	23.0	22.4	0.0	13.7	300.7
39	Aug-31	28.5	13.0	20.8	22.2	0.0	10.9	316.7
40	Sep-01	25.5	13.5	19.5	21.9	0.0	15.9	127.0
41	Sep-02	22.0	17.0	19.5	20.9	1.1	16.5	188.4
42	Sep-03	29.0	14.5	21.8	20.6	0.0	14.6	229.3
43	Sep-04	26.0	12.5	19.3	20.8	2.2	10.9	182.2
44	Sep-05	21.0	11.0	16.0	20.4	1.4	13.3	276.2
45	Sep-06	18.5	8.5	13.5	19.4	1.0	12.9	284.3
46	Sep-07	15.5	9.5	12.5	18.3	1.2	11.8	314.1
47	Sep-08	17.5	7.5	12.5	17.3	0.0	16.0	341.7
48	Sep-09	18.5	6.5	12.5	17.2	0.0	10.8	196.1
49	Sep-10	27.5	9.5	18.5	17.5	0.0	17.7	185.1
50	Sep-11	28.0	13.5	20.8	18.0	0.0	15.8	334.7
51	Sep-12	19.0	10.5	14.8	17.2	0.0	22.4	265.2
52	Sep-13	13.0	9.5	11.3	ND	5.6	8.7	332.3
53	Sep-14	16.5	4.5	10.5	ND	0.0	6.6	183.4
54	Sep-15	23.0	6.5	14.8	ND	0.0	15.1	177.1
55	Sep-16	16.5	10.0	13.3	ND	0.0	13.6	10.5
56	Sep-17	13.0	4.0	8.5	ND	0.6	14.1	10.1
57	Sep-18	14.0	-1.0	6.5	ND	1.8	14.3	191.7
58	Sep-19	12.0	7.0	9.5	ND	9.1	15.2	264.4
59	Sep-20	14.5	6.0	10.3	ND	0.2	15.7	323.5
60	Sep-21	9.0	2.0	5.5	ND	6.0	21.4	25.2
61	Sep-22	10.0	2.0	6.0	ND	0.0	15.2	327.6
62	Sep-23	13.0	-0.5	6.3	ND	0.2	11.4	244.5
63	Sep-24	11.5	4.5	8.0	ND	0.2	16.4	346.6
64	Sep-25	11.0	1.0	6.0	ND	1.1	9.2	322.5
65	Sep-26	14.5	2.0	8.3	12.8	0.0	6.8	339.3
66	Sep-27	20.0	2.0	11.0	13.1	0.0	9.3	205.1
67	Sep-28	22.5	6.5	14.5	13.3	0.0	6.1	298.4
68	Sep-29	23.5	7.5	15.5	13.2	0.0	10.0	177.6
69	Sep-30	25.0	9.0	17.0	12.8	0.0	15.4	181.7

Exp. Day	Date	Max Air Temp. (°C)	Min Air Temp. (°C)	Mean Air Temp. (°C)	Mean Water Temp. (°C)	Total Precip. (mm)	Max Wind Velocity (km/h)	Wind Direction (°)
70	Oct-01	18.0	9.0	13.5	10.5	0.0	13.9	341.8
71	Oct-02	19.5	5.0	12.3	7.8	0.0	23.6	188.8
72	Oct-03	15.0	7.5	11.3	6.9	14.0	11.4	359.3
73	Oct-04	4.5	1.0	2.8	6.6	25.8	19.2	316.9
74	Oct-05	3.0	-0.5	1.3	6.5	3.1	19.8	6.8
75	Oct-06	6.0	-1.5	2.3	6.1	0.0	10.6	280.5
76	Oct-07	7.0	-1.5	2.8	5.6	0.0	12.5	215.6
77	Oct-08	5.0	2.0	3.5	5.1	6.0	8.8	84.8
78	Oct-09	3.0	0.0	1.5	4.6	2.3	16.6	6.8
79	Oct-10	5.0	-1.0	2.0	4.7	0.3	10.4	262.2

Appendix B: ELA Nutrient Data

Table B.1. Total phosphorus (µg.L^{-1}) in mesocosms pre-treatment (June 11, 2012) and July 25 – October 2, 2012 post POEA treatment. ND = No data.

	Susp P (µg.L^{-1})							
	Pre-Treatment	Post Treatment						
	Jun-11	Jul-25	Jul-31	Aug-7	Aug-21	Sep-4	Sep-18	Oct-2
Lake	18	13	13	14	16	13	14	13
Control A	41	40	37	26	20	17	21	14
Control B	15	19	18	17	15	20	14	11
Low A	20	25	28	22	26	22	26	15
Low B	18	17	29	22	13	15	18	11
High A	11	9	15	13	12	14	14	11
High B	13	28	25	15	14	23	17	21
Control C	11	28	34	27	30	38	33	42
Low C	8	12	12	27	17	19	36	37
High C	9	10	9	11	14	23	19	22
	TDP (µg.L^{-1})							
Lake	7	4	3	2	4	3	5	2
Control A	8	5	7	7	6	6	6	6
Control B	11	11	8	8	8	7	8	4
Low A	8	8	9	5	6	8	7	7
Low B	12	12	15	10	9	7	6	5
High A	12	8	8	8	9	6	7	4
High B	8	6	5	6	7	5	7	5
Control C	6	7	ND	8	11	11	11	11
Low C	6	5	6	9	7	7	8	5
High C	5	3	4	5	5	6	5	6
	TP (µg.L^{-1})							
Lake	25	17	16	16	20	16	19	15
Control A	49	45	44	33	26	23	27	20
Control B	26	30	26	25	23	27	22	15
Low A	28	33	37	27	32	30	33	22
Low B	30	29	44	32	22	22	24	16
High A	23	17	23	21	21	20	21	15
High B	21	34	30	21	21	28	24	26
Control C	17	35	34	35	41	49	44	53
Low C	14	17	18	36	24	26	44	42
High C	14	13	13	16	19	29	24	28

ND: no data. Trophic Class: Mesotrophic Eutrophic (OECD, 1982)

Table B.2. Total nitrogen (µg.L^{-1}) pre-treatment (June 11, 2012) and July 25 – October 2, 2012 post POEA treatment. ND = No data.

	Susp N (µg.L^{-1})							
	Pre-Treatment	Post Treatment						
	Jun-11	Jul-25	Jul-31	Aug-7	Aug-21	Sep-4	Sep-18	Oct-2
Lake	380	296	319	297	368	339	358	288
Control A	768	560	339	251	292	270	504	197
Control B	163	132	284	231	288	444	357	197
Low A	236	336	311	236	365	211	493	276
Low B	292	133	386	409	195	227	428	180
High A	221	226	205	199	306	282	660	234
High B	125	340	294	163	161	314	430	284
Control C	155	351	380	419	444	460	557	367
Low C	190	185	199	257	160	270	449	342
High C	377	131	99	162	206	337	366	375
	TNP (µg.L^{-1})							
Lake	586	463	411	ND	701	593	481	464
Control A	517	473	445	438	473	530	511	597
Control B	914	1590	965	790	644	643	648	520
Low A	799	651	619	489	553	584	536	603
Low B	766	2266	1293	852	657	625	542	519
High A	626	1563	957	809	729	653	690	514
High B	513	537	460	497	524	516	572	515
Control C	494	487	ND	453	457	514	499	606
Low C	550	591	453	478	496	516	525	560
High C	708	477	429	470	496	526	536	544
	TN (µg.L^{-1})							
Lake	966	759	730	ND	1069	932	839	752
Control A	1285	1033	784	689	765	800	1015	794
Control B	1077	1722	1249	1021	932	1087	1005	717
Low A	1035	987	930	725	918	795	1029	879
Low B	1058	2399	1679	1261	852	852	970	699
High A	847	1789	1162	1008	1035	935	1350	748
High B	638	877	754	660	685	830	1002	799
Control C	649	838	ND	872	901	974	1056	973
Low C	740	776	652	735	656	786	974	902
High C	1085	608	528	632	702	863	902	919

ND: No data. Trophic class: Oligotrophic Mesotrophic Eutrophic Hypereutrophic (Galvez-Cloutier & Sanchez, 2007)

Table B.3. Total organic carbon (µg.L^{-1}) pre-treatment (June 11, 2012) and July 25 – October 2, 2012 post POEA treatment. ND = No data.

	POC (µg.L^{-1})							
	Pre-Treatment	Post Treatment						
	Jun-11	Jul-25	Jul-31	Aug-7	Aug-21	Sep-4	Sep-18	Oct-2
Lake	3410	3010	3220	3370	3580	6900	5250	6590
Control A	6740	4040	2540	1890	2340	4630	7060	2110
Control B	2350	1060	2070	2110	2350	7000	5850	2140
Low A	3240	2140	2350	2180	3270	2340	9930	3190
Low B	1930	1460	3150	3160	1440	4430	8050	3270
High A	1370	2030	1870	1900	2590	3440	13620	3390
High B	1700	2170	2040	1180	1210	3150	5170	3350
Control C	1810	2690	3660	4560	5140	6070	8710	4510
Low C	1130	1310	1400	1800	1240	4250	5880	3360
High C	1520	1040	980	1520	2070	5310	5380	5660
	DOC (µg.L^{-1})							
Lake	727	1327	721	85400	761	2478	1814	714
Control A	760	1008	900	974	1050	905	897	881
Control B	832	846	787	781	809	823	889	799
Low A	780	884	719	1155	784	895	943	933
Low B	797	795	833	776	763	739	751	756
High A	753	832	897	795	823	811	946	743
High B	826	729	715	910	736	759	874	789
Control C	814	728	88800	781	741	850	877	880
Low C	800	757	1147	937	773	723	932	865
High C	813	753	699	808	756	874	964	965
	TOC (µg.L^{-1})							
Lake	4137	4337	3941	88770	4341	9378	7064	7304
Control A	7500	5048	3440	2864	3390	5535	7957	2991
Control B	3182	1906	2857	2891	3159	7823	6739	2939
Low A	4020	3024	3069	3335	4054	3235	10873	4123
Low B	2727	2255	3983	3936	2203	5169	8801	4026
High A	2123	2862	2767	2695	3413	4251	14566	4133
High B	2526	2899	2755	2090	1946	3909	6044	4139
Control C	2624	3418	92460	5341	5881	6920	9587	5390
Low C	1930	2067	2547	2737	2013	4973	6812	4225
High C	2333	1793	1679	2328	2826	6184	6344	6625

Table B.4. Total Chlorophyll α (µg.L^{-1}) in mesocosms pre-treatment (June 11, 2012) and July 25 – October 2, 2012 post POEA treatment. ND = No data.

	Chl α (µg.L^{-1})							
	Pre-Treatment	Post Treatment						
	Jun-11	Jul-25	Jul-31	Aug-7	Aug-21	Sep-4	Sep-18	Oct-2
Lake	8	12	14	15	9	6	3	6
Control A	57	4	8	8	8	14	7	5
Control B	15	8	25	22	17	12	4	8
Low A	21	18	12	13	17	22	8	7
Low B	7	3	37	13	3	7	5	4
High A	4	7	11	12	17	11	12	4
High B	10	11	9	7	9	12	10	11
Control C	9	3	2	2	2	2	2	3
Low C	9	12	14	11	3	7	3	5
High C	3	4	5	4	4	10	3	5

Trophic Class: Oligotrophic | Mesotrophic | Eutrophic | Hypereutrophic

(OECD, 1982)

Appendix C: ELA Water Quality Data

Table C.1. Temperature (°C) in all mesocosms and Lake 114 from June 6 – October 11, 2012. ND = no data.

	Jun-6	Jun-11	Jun-12	Jun-19	Jun-21	Jun-26	Jul-11	Jul-18	Jul-23	Jul-24	Jul-25	Jul-27	Aug-15	Aug-21	Aug-31	Sep-6	Sep-11	Sep-18	Oct-3	Oct-11
LAKE	23.0	21.1	18.7	20.1	19.5	23.4	25.5	25.4	26.5	ND	ND	ND	21.7	19.5	22.0	18.8	18.9	14.2	12.3	5.0
Control A	22.7	20.6	18.1	19.6	18.8	22.7	25.0	24.6	26.2	26.2	27.0	21.8	21.2	19.0	22.0	18.3	18.6	14.0	12.2	4.9
Control B	22.8	20.4	18.2	19.7	19.0	23.5	25.0	25.0	26.4	26.2	27.2	21.7	21.1	18.9	22.5	18.2	18.3	14.1	12.0	4.5
Low A	23.1	20.5	18.0	19.6	18.7	23.0	25.0	24.9	26.3	26.2	27.1	22.0	21.2	19.0	22.0	18.2	18.8	14.1	12.0	4.4
Low B	22.8	20.5	18.2	19.6	19.0	23.3	24.9	24.8	26.3	26.3	27.1	21.7	21.1	19.0	22.2	18.2	18.6	13.9	12.0	4.6
High A	23.0	20.4	18.0	19.6	19.0	23.3	24.9	24.8	26.3	26.4	27.3	21.8	21.1	19.2	22.3	18.2	18.6	14.2	12.0	4.5
High B	22.8	20.3	18.3	19.7	18.9	23.6	25.0	25.0	26.4	26.1	27.2	21.9	21.2	19.0	22.3	18.3	18.9	14.1	12.1	4.5
Control C	22.5	20.5	18.3	19.8	19.1	23.2	25.2	24.6	26.0	26.3	27.0	21.9	21.3	19.1	22.1	18.4	18.3	14.0	12.1	4.8
Low C	22.8	20.7	18.3	19.8	19.0	23.3	25.1	24.7	26.1	26.2	27.1	22.1	21.3	19.1	22.0	18.4	18.9	14.0	12.0	4.7
High C	22.5	20.7	18.3	19.9	18.9	23.4	25.1	24.6	26.0	26.1	27.1	22.1	21.3	19.2	22.1	18.4	18.3	14.2	12.2	4.7

Table C.2. Specific conductivity (µS/cm) in all mesocosms and Lake 114 from June 6 – October 11, 2012. ND = no data.

	Jun-6	Jun-11	Jun-12	Jun-19	Jun-21	Jun-26	Jul-11	Jul-18	Jul-23	Jul-24	Jul-25	Jul-27	Aug-15	Aug-21	Aug-31	Sep-6	Sep-11	Sep-18	Oct-3	Oct-11
LAKE	8	8	8	8	8	9	8	8	9	ND	ND	ND	10	10	10	11	11	11	11	12
Control A	7	7	7	7	8	8	10	11	13	14	14	15	22	22	22	22	22	22	22	17
Control B	9	10	10	13	15	20	20	18	20	22	22	26	29	29	30	31	30	29	13	12
Low A	8	7	8	9	10	11	11	10	12	13	13	14	21	20	20	20	21	21	23	22
Low B	9	11	11	15	17	20	23	20	24	24	24	27	26	27	28	28	28	26	19	15
High A	9	11	11	15	17	20	20	16	18	19	18	20	23	22	24	25	25	22	12	12
High B	8	8	8	8	8	9	12	10	13	14	14	16	23	23	23	24	24	21	18	16
Control C	9	8	8	8	9	9	12	11	13	13	14	13	15	14	15	15	15	16	17	17
Low C	9	8	8	8	9	9	11	10	12	12	12	13	15	15	16	17	17	16	15	14
High C	9	9	8	9	9	8	10	9	11	12	11	12	14	13	14	14	15	16	16	16

Table C.3. Dissolved oxygen ($mg.L^{-1}$) in all mesocosms and Lake 114 from June 6 – October 11, 2012. ND = no data.

	Jun-6	Jun-11	Jun-12	Jun-19	Jun-21	Jun-26	Jul-11	Jul-18	Jul-23	Jul-24	Jul-25	Jul-27	Aug-15	Aug-21	Aug-31	Sep-6	Sep-11	Sep-18	Oct-3	Oct-11
LAKE	7.6	6.9	7.7	5.3	7.2	7.8	7.1	7.7	7.7	ND	ND	ND	7.3	6.7	7.1	7.4	7.8	9.7	9.0	10.6
Control A	8.8	7.8	10.2	8.9	8.7	7.9	6.1	6.3	5.3	5.1	4.8	5.3	6.0	6.3	7.0	6.1	7.9	8.0	7.9	10.1
Control B	8.4	6.8	6.2	4.4	3.3	3.4	7.0	8.0	7.1	7.3	7.2	7.0	6.2	6.8	6.4	6.9	6.8	7.5	7.8	10.4
Low A	8.1	7.4	8.4	6.9	6.1	7.1	7.2	7.9	7.8	7.3	7.6	7.1	6.8	6.3	6.5	7.5	7.6	7.6	8.0	10.3
Low B	7.9	7.9	4.4	3.5	2.2	3.5	6.5	8.2	7.9	7.2	7.8	6.6	5.4	6.3	5.6	6.5	7.4	7.6	7.6	10.1
High A	7.5	3.8	4.7	8.3	2.6	3.7	7.2	8.7	8.2	7.9	7.7	6.7	6.0	6.3	6.4	7.3	7.6	8.9	8.0	10.6
High B	7.6	7.2	8.2	7.6	7.2	6.8	6.8	8.0	7.1	6.8	6.5	6.3	4.7	6.0	6.5	6.8	7.4	8.5	8.1	10.7
Control C	8.2	7.5	8.2	7.4	7.3	7.2	6.8	8.3	7.3	7.7	7.6	7.0	6.6	6.3	6.2	7.7	7.3	7.8	7.5	10.5
Low C	7.1	7.9	8.2	7.2	6.8	7.8	6.7	7.3	6.5	6.2	7.4	7.3	5.1	6.0	7.0	7.8	7.6	8.3	8.5	10.6
High C	7.8	6.8	7.5	7.5	7.9	7.4	7.0	7.7	6.8	7.0	7.6	6.6	6.6	6.5	7.0	7.3	6.9	8.4	7.5	10.6

Table C.4. pH in all mesocosms and Lake 114 from June 6 – October 11, 2012. ND = no data.

	Jun-6	Jun-11	Jun-12	Jun-19	Jun-21	Jun-26	Jul-11	Jul-18	Jul-23	Jul-24	Jul-25	Jul-27	Aug-15	Aug-21	Aug-31	Sep-6	Sep-11	Sep-18	Oct-3	Oct-11
LAKE	6.1	5.0	5.1	5.3	5.6	5.8	5.8	6.9	6.7	ND	ND	ND	6.8	6.2	5.5	6.1	7.3	4.9	5.4	5.4
Control A	6.5	5.3	5.3	5.4	5.5	5.4	5.0	5.5	5.4	5.5	5.3	5.1	6.1	5.5	5.6	6.0	7.3	5.2	5.1	5.2
Control B	6.3	5.1	5.0	5.3	5.4	5.8	5.9	6.8	7.1	6.5	6.7	6.0	7.0	6.6	6.2	6.3	7.4	5.3	5.2	5.2
Low A	6.1	5.1	5.0	4.9	4.8	4.8	5.3	6.4	5.9	6.2	6.9	6.1	6.8	6.4	6.5	6.7	7.9	5.7	5.5	5.4
Low B	6.1	5.0	5.0	5.3	5.6	5.8	5.9	7.0	7.2	7.1	7.3	6.4	7.1	6.6	6.2	6.3	7.3	5.5	5.3	5.2
High A	5.9	5.0	4.9	5.2	5.6	5.5	5.5	7.2	6.8	7.3	7.7	6.4	7.6	6.8	6.6	6.9	8.1	6.5	5.5	5.4
High B	6.3	5.2	5.2	5.2	5.5	5.9	5.7	6.7	6.8	6.9	6.5	6.0	6.5	6.3	6.4	6.5	7.7	5.8	5.6	5.5
Control C	6.2	5.3	5.2	5.4	5.8	5.8	5.8	6.8	6.6	6.3	6.3	5.7	6.3	5.8	5.9	6.5	7.5	5.2	5.3	5.3
Low C	6.2	5.3	5.3	5.5	5.6	5.9	5.7	6.5	6.8	6.7	6.9	6.0	6.5	6.1	6.2	6.5	7.7	5.6	5.6	5.5
High C	6.2	5.2	5.4	5.4	5.4	5.9	5.8	6.5	6.7	6.5	6.7	6.0	7.1	6.2	6.5	6.7	7.9	7.7	5.6	5.5

Table C.5. Oxidation-reduction potential (mV) in all mesocosms and Lake 114 from June 6 – October 11, 2012. ND = no data.

	Jun-19	Jun-21	Jun-26	Jul-11	Jul-18	Jul-23	Jul-24	Jul-25	Jul-27	Aug-15	Aug-21	Aug-31	Sep-6	Sep-11	Sep-18	Oct-3	Oct-11
Lake	349	163	182	149	164	118	ND	ND	ND	157	161	173	162	145	169	145	179
Control A	335	151	143.00	157	203	160	124	93	151	133	143	158	121	133	158	124	158
Control B	346	167	173	136	175	101	91	63	128	120	130	149	143	128	155	129	166
Low A	339	176	141	155	170	131	103	97	136	146	147	150	149	98	143	126	168
Low B	346	161	167	138	169	90	79	55	121	132	143	154	159	135	152	133	171
High A	346	159	162	128	156	89	78	70	125	117	121	156	154	96	125	137	177
High B	350	176	182	141	180	127	109	119	144	157	139	163	161	119	148	129	172
Control C	340	149	181	146	158	126	99	79	139	137	145	153	142	131	168	130	162
Low C	340	167	183	142	185	124	107	93	143	159	158	169	160	122	156	127	169
High C	341	159	185	147	180	125	111	109	143	147	152	164	165	132	153	137	178

Appendix D: ELA Fish Data

Table D.1. Fork length and weight of pre-exposure subset of Fathead minnows from Lake 114 on July 23, 2012. ND = No data.

Exposure	Treatment	Fish #	Fork Length (cm)	Wt (g)	Sex (M/F)	Fulton's Condition
Pre-exposure	Lake 114	1	5.5	2.5	M	1.50
Pre-exposure	Lake 114	2	5.5	2.6	M	1.56
Pre-exposure	Lake 114	3	5.8	2.8	M	1.44
Pre-exposure	Lake 114	4	5.6	2.5	M	1.42
Pre-exposure	Lake 114	5	5.6	2.3	M	1.31
Pre-exposure	Lake 114	6	5.5	1.9	M	1.14
Pre-exposure	Lake 114	7	5.6	2.1	M	1.20
Pre-exposure	Lake 114	8	5.7	2.8	M	1.51
Pre-exposure	Lake 114	9	5.7	2.2	M	1.19
Pre-exposure	Lake 114	10	5.9	3.1	M	1.51
Mean			5.64	2.48		1.38
SD			0.13	0.36		0.16
Pre-exposure	Lake 114	11	6.1	2.7	F	1.19
Pre-exposure	Lake 114	12	5.5	2.1	F	1.26
Pre-exposure	Lake 114	13	4.5	1.1	F	1.21
Pre-exposure	Lake 114	14	5.5	2.2	F	1.32
Pre-exposure	Lake 114	15	4.9	1.8	F	1.53
Pre-exposure	Lake 114	16	6	3.1	F	1.44
Pre-exposure	Lake 114	17	5.1	2.1	F	1.58
Pre-exposure	Lake 114	18	5.2	2.2	F	1.56
Pre-exposure	Lake 114	19	5.4	2.2	F	1.40
Pre-exposure	Lake 114	20	5.5	2.0	F	1.20
Mean			5.37	2.15		1.37
SD			0.48	0.52		0.15

Table D.2. Fork length and weight of Fathead minnows in all mesocosms after 1 week POEA exposure from July 24 – 31, 2012. ND = No data.

Fork Length (cm)									
Fish	Control A	Control B	Low A	Low B	High A	High B	Control C	Low C	High C
1	5.3	7.2	5.2	6.2	5.8	6.1	7.1	6.0	6.0
2	5.4	5.8	5.7	7.1	5.9	5.9	5.2	5.7	5.7
3	5.9	6.0	5.9	5.7	6.2	5.9	6.7	5.9	6.1
4	6.2	5.9	6.0	5.7	5.7	5.7	5.7	5.8	6.0
5	5.8	5.7	5.3	5.0	5.9	5.7	5.7	5.8	5.7
6	5.7	5.8	6.0	6.0	5.8	5.8	4.8	5.8	6.5
7	5.8	6.0	5.8	6.2	5.4	5.9	5.8	5.7	5.2
8	ND	6.0	4.7	5.5	5.0	5.9	5.6	ND	6.2
9	ND	5.8	ND	5.9	5.9	ND	5.7	ND	5.9
10	ND	ND	ND	5.8	ND	ND	6.0	ND	5.8
Mean	5.7	6.0	5.6	5.9	5.7	5.9	5.8	5.8	5.9
SD	0.30	0.45	0.47	0.55	0.35	0.13	0.66	0.11	0.35
Weight (g)									
1	1.6	5.5	1.7	3.5	2.6	3.1	4	3.3	3.2
2	1.9	2.8	2.1	4.8	2.7	2.8	1.6	2	2
3	2.4	3.3	2.2	2.4	3.5	2.8	2.9	3	3.2
4	2.5	3.2	2.4	2.6	2.8	2.1	2.1	2.8	3.6
5	2.1	2.5	1.7	1.6	3.1	2.5	2.3	3	2.9
6	2.4	2.4	2.9	2.7	3	2.8	1.1	2.3	3.2
7	2.3	3.3	2.6	2.6	2.3	2.8	1.7	2.8	1.8
8	ND	3	1.4	2	1.8	2.3	2.1	ND	3.7
9	ND	2.1	ND	2	3.2	ND	2.3	ND	2.2
10	ND	ND	ND	1.8	ND	ND	2.3	ND	2
Mean	2.2	3.1	2.1	2.6	2.8	2.7	2.2	2.7	2.8
SD	0.33	0.99	0.51	0.95	0.51	0.33	0.79	0.45	0.71

Table D.3. Fork length and weight of Fathead minnows in all mesocosms after 2 week POEA exposure from July 31 - August 14, 2012. ND = No data.

Fork Length (cm)									
Fish	Control A	Control B	Low A	Low B	High A	High B	Control C	Low C	High C
1	6.4	6.0	5.8	6.0	5.6	6.0	6.3	5.8	5.6
2	5.9	6.2	4.9	6.3	5.2	6.7	5.3	5.7	5.7
3	5.7	5.7	4.8	5.5	5.2	5.5	6.1	6.9	5.7
4	5.3	6.0	5.3	6.0	5.6	6.0	5.5	5.8	5.5
5	5.2	6.2	4.8	5.8	6.0	4.8	ND	5.5	5.1
6	5.8	5.9	6.1	5.5	5.5	5.1	ND	5.7	6.6
7	ND	5.7	5.7	ND	5.6	5.4	ND	5.6	6.7
8	ND	ND	5.8	ND	5.7	5.2	ND	5.5	6.1
9	ND	ND	5.3	ND	6.1	ND	ND	ND	ND
10	ND	ND	ND	ND	5.5	ND	ND	ND	ND
Mean	5.7	6.0	5.4	5.9	5.6	5.6	5.8	5.8	5.9
SD	0.4	0.2	0.5	0.3	0.3	0.6	0.5	0.5	0.6
Weight (g)									
1	3.5	3.2	3.1	2.1	2.6	2.2	3	3.1	2.8
2	2.8	3.5	1.6	1.9	1.5	3.1	2	2.8	2
3	3.1	3.1	1.7	1.9	1.5	2	2.8	3.3	2.8
4	2	3.5	2.4	2.7	2.4	2.7	2.3	2.3	1.9
5	2.1	3.1	1.3	2.6	2.5	1.5	ND	2.7	1.9
6	2.2	2.4	2.2	1.8	1.9	1.7	ND	2.6	2.7
7	ND	2.5	3	ND	2.2	1.6	ND	2.5	2.9
8	ND	ND	2.3	ND	2.5	1.8	ND	2.3	2.7
9	ND	ND	1.5	ND	2.2	ND	ND	ND	ND
10	ND	ND	ND	ND	2	ND	ND	ND	ND
Mean	2.6	3.0	2.1	2.2	2.1	2.1	2.5	2.7	2.5
SD	0.61	0.44	0.65	0.39	0.40	0.57	0.46	0.36	0.44

Table D.4. Fork length and weight of Fathead minnows in all mesocosms after 4 week POEA exposure from August 14 - September 11, 2012. ND = No data.

Fork Length (cm)									
Fish	Control A	Control B	Low A	Low B	High A	High B	Control C	Low C	High C
1	6.2	6.0	6	6.2	5.7	6.0	6.4	6.0	5.4
2	6.4	6.5	5.8	6.2	6.0	7.1	5.9	6.2	6.0
3	6.0	5.7	6.1	6.0	6.0	5.8	5.7	6.6	5.6
4	5.5	6.2	5.6	5.9	6.5	6.1	6.2	6.1	6.0
5	6.1	5.9	6.0	ND	6.0	5.2	6.2	6.0	5.8
6	6.2	6.1	5.6	ND	6.2	6.5	5.8	6.0	5.7
7	6.0	5.8	6.0	ND	5.8	6.1	6.0	6.0	6.0
8	5.6	6.2	6.2	ND	5.7	6.2	5.7	ND	5.5
9	5.8	5.8	6.6	ND	6.4	7.4	5.8	ND	5.7
10	ND	ND	ND	ND	5.9	ND	ND	ND	ND
Mean	6.0	6.0	6.0	6.1	6.0	6.3	6.0	6.1	5.7
SD	0.29	0.25	0.31	0.15	0.27	0.66	0.25	0.22	0.22
Weight (g)									
1	3.2	2	2.4	2.5	2.4	3.1	2.5	2.5	1.8
2	3.3	3.6	2.7	2.6	2.7	3.8	2.3	2.8	2.5
3	2.4	2.7	2.5	1.9	2.5	2	2.1	3.1	2.2
4	1.8	3	2.2	2.3	3	2.6	2.8	2.6	1.9
5	2.9	1.9	2.5	ND	1.9	1.6	2.9	2.4	2
6	2.7	2.4	2	ND	2.9	3	1.7	2.4	2.1
7	2.3	2	2.6	ND	2.7	2.3	2.4	2.5	2.6
8	1.8	2.8	2.7	ND	2.1	3	1.5	ND	1.8
9	2.1	2.4	3.3	ND	3.1	4.6	1.9	ND	2.2
10	ND	ND	ND	ND	2.6	ND	ND	ND	ND
Mean	2.5	2.5	2.5	2.3	2.6	2.9	2.2	2.6	2.1
SD	0.56	0.55	0.36	0.31	0.38	0.92	0.48	0.25	0.29

Appendix E: Laboratory Water Quality Data

Table E.1: Water quality parameters monitored in each POEA exposure tank over the 7 day experiment from October 21 – 27, 2013.

Treatment	Date	pH	Temp (°C)	DO%	DO ($mg.L^{-1}$)	NH_4^+ ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)
Control A	21-Oct-13	7.63	19.0	84	7.8	0.6	0.011
Control A	22-Oct-13	7.62	18.1	84	8.0	2.4	0.041
Control A	23-Oct-13	7.70	17.9	86	8.2	1.2	0.024
Control A	24-Oct-13	7.83	18.0	85	8.1	0.6	0.016
Control A	25-Oct-13	7.71	18.2	78	7.3	2.4	0.051
Control A	26-Oct-13	7.70	18.1	83	7.9	1.2	0.025
Control A	27-Oct-13	7.71	18.0	82	7.8	1.2	0.025
Control B	21-Oct-13	7.44	19.1	83	7.9	0.6	0.007
Control B	22-Oct-13	7.71	17.8	85	8.1	1.2	0.025
Control B	23-Oct-13	7.74	17.8	84	8.0	1.2	0.026
Control B	24-Oct-13	7.90	17.8	86	8.2	1.2	0.038
Control B	25-Oct-13	7.89	18.0	81	7.6	2.4	0.075
Control B	26-Oct-13	7.74	17.9	83	7.9	2.4	0.053
Control B	27-Oct-13	7.80	17.9	79	7.6	2.4	0.061
Control C	21-Oct-13	7.97	19.4	82	7.8	0.6	0.025
Control C	22-Oct-13	7.82	18.3	83	7.8	2.4	0.066
Control C	23-Oct-13	7.83	18.5	79	7.4	1.2	0.034
Control C	24-Oct-13	7.58	18.5	80	7.5	1.2	0.019
Control C	25-Oct-13	7.93	18.5	75	7.0	2.4	0.085
Control C	26-Oct-13	7.83	18.4	78	7.4	1.2	0.034
Control C	27-Oct-13	7.80	18.4	75	7.1	2.4	0.063
Control D	21-Oct-13	7.99	18.0	85	8.2	0.6	0.024
Control D	22-Oct-13	7.76	16.7	84	8.1	0.6	0.013
Control D	23-Oct-13	7.90	16.6	87	8.5	0.6	0.017
Control D	24-Oct-13	7.95	16.7	83	8.1	0.6	0.020
Control D	25-Oct-13	7.89	17.8	82	7.8	2.4	0.074
Control D	26-Oct-13	7.90	16.9	83	8.1	1.2	0.036
Control D	27-Oct-13	7.88	16.5	81	8.0	1.2	0.033
Control Stock	21-Oct-13	8.05	19.0	85	8.1	0	0
Control Stock	22-Oct-13	7.88	17.3	86	8.3	0	0
Control Stock	23-Oct-13	7.95	17.8	81	7.7	0	0
Control Stock	24-Oct-13	7.92	17.9	81	7.7	0	0
Control Stock	25-Oct-13	7.93	17.7	82	8.0	0	0
Low A	21-Oct-13	6.88	18.9	84	7.9	2.4	0.008
Low A	22-Oct-13	7.44	18.0	82	7.8	2.4	0.027
Low A	23-Oct-13	7.44	18.0	82	7.8	1.2	0.014
Low A	24-Oct-13	7.84	18.0	81	7.7	1.2	0.034
Low A	25-Oct-13	7.62	17.8	80	7.6	2.4	0.040

Treatment	Date	pH	Temp (°C)	DO%	DO ($mg.L^{-1}$)	NH_4^+ ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)
Low A	26-Oct-13	7.44	18.0	80	7.7	2.4	0.027
Low A	27-Oct-13	7.56	17.9	77	7.4	2.4	0.035
Low B	21-Oct-13	7.73	19.5	85	7.9	1.2	0.029
Low B	22-Oct-13	7.8	18.2	80	7.6	2.4	0.062
Low B	23-Oct-13	7.86	17.6	82	7.7	2.4	0.068
Low B	24-Oct-13	7.96	18.5	78	7.4	2.4	0.091
Low B	25-Oct-13	7.94	18.0	79	7.5	2.4	0.084
Low B	26-Oct-13	7.86	18.1	78	7.5	1.2	0.035
Low B	27-Oct-13	7.88	18.4	75	7.1	2.4	0.076
Low C	21-Oct-13	7.92	19.6	82	7.6	0.6	0.023
Low C	22-Oct-13	7.72	18.4	81	7.6	2.4	0.053
Low C	23-Oct-13	7.80	18.6	76	7.1	1.2	0.032
Low C	24-Oct-13	7.62	18.8	69	6.4	1.2	0.022
Low C	25-Oct-13	7.94	18.3	79	7.4	2.4	0.086
Low C	26-Oct-13	7.80	18.6	76	7.2	2.4	0.064
Low C	27-Oct-13	7.78	18.7	74	7.0	2.4	0.062
Low D	21-Oct-13	7.96	17.9	82	7.9	0.6	0.022
Low D	22-Oct-13	7.76	16.6	85	8.3	1.2	0.025
Low D	23-Oct-13	7.88	16.8	82	8.0	1.2	0.034
Low D	24-Oct-13	7.94	16.5	85	8.3	1.2	0.038
Low D	25-Oct-13	7.86	16.8	83	8.0	2.4	0.064
Low D	26-Oct-13	7.88	16.6	83	8.1	2.4	0.066
Low D	27-Oct-13	7.86	16.3	81	8.0	2.4	0.062
Low Stock	21-Oct-13	8.07	17.9	85	8.3	0	0
Low Stock	22-Oct-13	7.89	16.3	88	8.6	0	0
Low Stock	23-Oct-13	7.95	16.6	86	8.4	0	0
Low Stock	24-Oct-13	7.90	16.6	84	8.2	0	0
Low Stock	25-Oct-13	7.92	16.9	81	8.4	0	0
High A	21-Oct-13	6.84	19.1	81	7.7	1.2	0.004
High A	22-Oct-13	7.67	17.8	81	7.7	2.4	0.045
High A	23-Oct-13	7.52	17.6	82	7.9	2.4	0.032
High A	24-Oct-13	7.83	17.6	81	7.8	2.4	0.064
High A	25-Oct-13	7.77	17.7	75	7.1	2.4	0.056
High A	26-Oct-13	7.52	17.6	78	7.6	1.2	0.016
High A	27-Oct-13	7.66	17.7	75	7.3	2.4	0.044
High B	21-Oct-13	7.71	19.2	80	7.6	0.6	0.014
High B	22-Oct-13	7.76	17.9	81	7.7	1.2	0.028
High B	23-Oct-13	7.82	18.0	81	7.7	1.2	0.032
High B	24-Oct-13	7.96	18.5	81	7.7	1.2	0.046
High B	25-Oct-13	7.85	18.0	79	7.4	2.4	0.069
High B	26-Oct-13	7.82	18.1	79	7.5	2.4	0.065
High B	27-Oct-13	7.84	18.0	74	7.1	1.2	0.034
High C	21-Oct-13	7.96	18.1	86	8.2	1.2	0.044

Treatment	Date	pH	Temp (°C)	DO%	DO ($mg.L^{-1}$)	NH_4^+ ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)
High C	22-Oct-13	7.88	16.4	86	8.4	1.2	0.033
High C	23-Oct-13	7.91	16.7	86	8.4	0.6	0.018
High C	24-Oct-13	7.91	16.5	82	8.1	0.6	0.018
High C	25-Oct-13	7.90	16.9	82	7.9	2.4	0.071
High C	26-Oct-13	7.91	16.5	83	8.2	1.2	0.035
High C	27-Oct-13	7.90	16.1	79	7.9	1.2	0.034
High D	21-Oct-13	7.88	17.9	84	8.1	0.6	0.018
High D	22-Oct-13	7.75	16.3	86	8.5	2.4	0.048
High D	23-Oct-13	7.84	16.7	85	8.3	2.4	0.061
High D	24-Oct-13	7.87	16.2	84	8.3	1.2	0.032
High D	25-Oct-13	7.85	16.7	82	7.9	2.4	0.063
High D	26-Oct-13	7.84	16.3	83	8.2	1.2	0.030
High D	27-Oct-13	7.83	15.8	81	8.0	1.2	0.028
High Stock	21-Oct-13	8.15	18.6	85	8.2	0	0
High Stock	22-Oct-13	7.87	16.7	85	8.3	0	0
High Stock	23-Oct-13	8.0	17.3	83	8.0	0	0
High Stock	24-Oct-13	8.01	17.2	81	7.9	0	0
High Stock	25-Oct-13	7.96	17.0	83	8.1	0	0

Appendix F: Laboratory Data – Histopathological and Biochemical Results

Table F.1. Data collection on Fathead minnows exposed to POEA for the 7 day duration of the experiment from October 21 – 27, 2013. (FCF: Fulton's condition factor; PAGE: gill portion available for gas exchange; TBARS: thiobarbituric reactive substances). ND = no data.

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS ($nmol.mg\ protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
Pre-exposure	0A2C1	0.61	4.1	M	0.89	32.8	ND	1.86	0.44	ND	7.17	3.04
Pre-exposure	0A2C2	2.21	6.0	F	1.02	54.9	21	1.66	0.53	355	7.17	3.04
Pre-exposure	0A4C1	2.72	5.5	F	1.63	38.8	21	1.13	0.89	560	19.44	5.89
Pre-exposure	0A4C2	1.06	5	M	0.84	44.8	17	1.02	0.79	615	19.44	5.89
Pre-exposure	0A8C1	1.37	5.5	F	0.82	44.0	18	5.86	0.27	500	18.95	4.38
Pre-exposure	0A8C2	0.73	4.4	F	0.86	35.7	24	3.04	0.53	520	18.95	4.38
Pre-exposure	0A12C1	0.84	4.5	F	0.92	31.9	21	0.80	0.66	499	3.52	10.07
Pre-exposure	0A12C2	0.73	4.4	F	0.86	48.2	19	2.03	0.15	442	3.52	10.07
Pre-exposure	0A1L1	1.99	5.7	F	1.08	42.8	15	0.80	0.73	535	13.44	5.58
Pre-exposure	0A1L2	2.20	5.9	M	1.07	ND	ND	0.44	2.85	421	13.44	5.58
Pre-exposure	0A6L1	1.46	5.3	M	0.98	43.0	25	1.25	1.02	558	14.26	3.49
Pre-exposure	0A6L2	0.82	4.6	F	0.84	41.8	18	2.95	0.47	ND	14.26	3.49
Pre-exposure	0A7L1	1.90	5.8	F	0.97	44.7	21	0.77	1.10	532	0.72	4.81
Pre-exposure	0A7L2	1.22	5.0	F	0.98	36.8	21	1.21	0.91	618	2.99	2.02
Pre-exposure	0A11L1	3.35	6.2	M	1.41	49.6	18	0.86	3.70	454	19.89	2.51
Pre-exposure	0A11L2	1.35	5.2	F	0.96	49.1	21	0.90	1.22	343	11.12	8.32
Pre-exposure	0A3H1	2.40	6.0	M	1.11	47.6	22	0.32	1.46	456	15.31	4.95
Pre-exposure	0A3H2	0.58	4.0	J	0.90	ND	ND	1.86	0.34	459	21.31	5.89
Pre-exposure	0A5H1	2.24	5.8	F	1.15	44.8	22	1.11	1.30	514	1.49	4.20

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
Pre-exposure	0A5H2	0.82	4.6	F	0.84	47.4	25	0.97	0.86	695	25.23	5.92
Pre-exposure	0A9H1	2.80	6.4	M	1.07	47.4	18	1.18	2.00	554	1.37	7.04
Pre-exposure	0A9H2	1.64	5.3	F	1.10	30.5	26	1.63	0.70	512	0.60	4.64
Pre-exposure	0A10H1	1.50	5.6	F	0.86	31.7	22	0.68	0.70	ND	28.91	3.21
Pre-exposure	0A10H2	1.31	5	M	1.05	35.2	22	0.12	1.29	413	2.89	5.21
2 Day Control	2A2C1	3.28	6.5	M	1.19	ND	ND	1.04	1.97	643	20.99	8.18
2 Day Control	2A2C2	1.12	5.7	F	0.60	ND	ND	1.03	0.83	352	13.23	5.93
2 Day Control	2A2C3	1.27	4.9	F	1.08	47.9	21	0.86	1.40	657	13.23	5.93
2 Day Control	2A2C4	0.65	4.2	F	0.88	ND	ND	0.28	1.31	ND	13.23	5.93
2 Day Control	2A2C5	1.67	5	F	1.34	41.9	23	1.15	1.37	276	0.72	7.36
2 Day Control	2A2C6	0.81	4.2	F	1.09	ND	ND	0.49	0.33	ND	13.23	6.28
2 Day Control	2A4C1	0.82	4.6	F	0.84	ND	ND	1.39	0.54	ND	10.68	5.56
2 Day Control	2A4C2	0.73	4.2	F	0.99	ND	ND	1.37	0.95	ND	1.92	9.14
2 Day Control	2A4C3	1.58	5.1	F	1.19	26.0	10	0.78	0.47	426	10.68	5.56
2 Day Control	2A4C4	3.42	6.7	M	1.14	51.0	17	4.63	0.42	500	6.40	3.14
2 Day Control	2A4C5	0.76	4.3	J	0.96	ND	ND	1.26	0.75	658	10.68	5.56
2 Day Control	2A4C6	2.98	6.4	J	1.14	ND	ND	3.49	0.61	519	1.92	9.14
2 Day Control	2A8C1	3.12	6.7	M	1.04	ND	ND	0.82	3.77	ND	13.21	6.20
2 Day Control	2A8C2	0.82	4.5	J	0.90	ND	ND	0.86	0.72	ND	12.23	5.74
2 Day Control	2A8C3	1.07	5	M	0.85	41.9	15	1.66	0.73	431	16.68	6.67
2 Day Control	2A8C4	3.61	6.8	M	1.15	50.4	15	1.06	3.43	391	15.12	7.36
2 Day Control	2A8C5	2.17	6	F	1.01	ND	ND	1.16	1.81	503	16.68	6.67
2 Day Control	2A8C6	1.09	5.1	F	0.82	ND	ND	3.44	0.26	857	12.23	5.74
2 Day Control	2A12C1	2.03	5.7	F	1.10	50.7	16	0.62	1.73	313	8.85	7.83
2 Day Control	2A12C2	1.05	4.9	F	0.89	ND	ND	0.76	0.63	422	19.23	6.92

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
2 Day Control	2A12C3	1.54	5.2	F	1.09	52.8	18	0.11	0.93	703	19.23	6.92
2 Day Control	2A12C4	1.49	5.3	F	1.00	ND	ND	0.46	1.16	ND	22.63	8.18
2 Day Control	2A12C5	1.60	5.3	F	1.07	ND	ND	0.65	1.51	366	0.66	6.82
2 Day Control	2A12C6	0.75	4.5	F	0.83	ND	ND	0.22	1.00	ND	8.85	7.83
2 Day Low	2A1L1	0.80	4.5	F	0.87	29.7	21	1.23	1.29	627	16.54	4.74
2 Day Low	2A1L2	0.77	4.3	F	0.97	ND	ND	0.84	0.63	ND	16.54	4.74
2 Day Low	2A1L3	1.26	4.8	F	1.14	ND	ND	0.72	1.08	709	7.15	5.68
2 Day Low	2A1L4	1.52	5.6	F	0.87	ND	ND	1.39	0.94	ND	0.98	6.53
2 Day Low	2A1L5	2.59	6.5	M	0.94	ND	ND	1.46	2.05	ND	20.76	4.18
2 Day Low	2A1L6	3.95	7	M	1.15	30.8	15	0.82	4.18	ND	0.65	5.71
2 Day Low	2A6L1	0.87	4.4	F	1.02	ND	ND	1.69	0.74	ND	0.61	6.04
2 Day Low	2A6L2	0.84	4.5	J	0.92	31.7	28	0.56	1.22	538	17.57	5.73
2 Day Low	2A6L3	1.20	5.3	M	0.80	ND	ND	1.91	0.70	ND	8.98	7.33
2 Day Low	2A6L4	1.76	5.5	F	1.06	ND	ND	0.14	2.85	ND	15.87	5.83
2 Day Low	2A6L5	0.69	4.3	J	0.87	ND	ND	1.10	1.43	ND	12.42	6.02
2 Day Low	2A6L6	1.64	5.5	M	0.99	30.9	18	0.58	1.44	435	17.57	5.73
2 Day Low	2A7L1	1.73	5.6	F	0.98	36.8	26	1.25	0.76	605	22.03	5.38
2 Day Low	2A7L2	3.03	6.6	F	1.06	37.5	24	1.10	0.93	428	2.59	4.25
2 Day Low	2A7L3	1.82	5.7	F	0.98	ND	ND	1.25	1.38	ND	22.03	5.38
2 Day Low	2A7L4	1.65	5.4	M	1.05	ND	ND	1.10	0.93	ND	2.59	4.25
2 Day Low	2A7L5	0.76	4	F	1.18	ND	ND	0.28	0.62	ND	15.98	6.13
2 Day Low	2A7L6	0.94	4.2	F	1.26	ND	ND	1.32	0.97	ND	15.98	6.13
2 Day Low	2A11L1	1.25	4.9	F	1.07	ND	ND	0.86	0.84	ND	10.62	4.32
2 Day Low	2A11L2	3.23	6.3	M	1.29	58.5	12	0.77	2.20	ND	30.37	5.89
2 Day Low	2A11L3	1.83	5.4	F	1.16	57.0	25	0.65	0.85	608	28.63	3.80

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
2 Day Low	2A11L4	1.64	5.1	F	1.24	ND	ND	0.37	2.07	ND	10.62	4.32
2 Day Low	2A11L5	1.77	5.1	F	1.34	ND	ND	1.40	1.96	ND	13.51	6.86
2 Day Low	2A11L6	1.10	4.9	F	0.94	ND	ND	1.54	0.31	ND	6.97	7.37
2 Day High	2A3H1	2.91	6.4	M	1.11	42.8	26	1.20	2.06	531	28.79	6.83
2 Day High	2A3H2	1.87	5.6	F	1.07	ND	ND	0.11	2.18	ND	15.12	5.72
2 Day High	2A3H3	0.56	3.8	J	1.02	ND	ND	0.67	0.65	ND	11.42	6.33
2 Day High	2A3H4	0.89	4.5	F	0.97	ND	ND	0.53	1.31	ND	0.52	6.18
2 Day High	2A3H5	1.32	5.2	F	0.94	49.5	16	0.33	1.28	707	0.76	3.32
2 Day High	2A3H6	1.77	5.3	F	1.19	ND	ND	0.98	1.08	ND	11.42	6.33
2 Day High	2A5H1	1.59	5.2	F	1.13	44.5	19	1.07	1.05	ND	13.87	8.03
2 Day High	2A5H2	1.05	4.8	F	0.95	ND	ND	0.37	0.26	ND	19.12	4.95
2 Day High	2A5H3	1.15	4.8	F	1.04	ND	ND	1.19	1.15	ND	15.22	7.01
2 Day High	2A5H4	1.18	5	F	0.95	24.4	15	1.30	0.71	357	6.26	4.95
2 Day High	2A5H5	1.83	5.5	F	1.10	ND	ND	0.06	0.77	650	11.38	5.97
2 Day High	2A5H6	1.12	4.9	F	0.95	ND	ND	1.20	0.92	ND	13.87	8.03
2 Day High	2A9H1	2.19	6		1.01	32.8	23	1.40	1.19	457	10.18	7.19
2 Day High	2A9H2	0.82	4.3	F	1.03	28.9	19	0.44	1.51	627	7.96	6.03
2 Day High	2A9H3	0.73	4.1	F	1.06	ND	ND	0.63	0.99	ND	7.96	6.03
2 Day High	2A9H4	1.11	5	F	0.89	ND	ND	0.21	0.44	ND	7.96	6.03
2 Day High	2A9H5	0.97	4.8		0.87	ND	ND	0.63	0.93	ND	7.46	4.27
2 Day High	2A9H6	0.57	4	F	0.88	ND	ND	0.62	1.07	ND	7.46	4.27
2 Day High	2A10H1	0.78	4.2	F	1.06	ND	ND	0.49	2.72	ND	5.45	5.54
2 Day High	2A10H2	1.07	6	M	0.49	ND	ND	1.24	1.69	ND	3.14	5.71
2 Day High	2A10H3	1.46	5.2	F	1.04	ND	ND	0.50	1.07	463	5.45	5.54
2 Day High	2A10H4	1.05	4.8	F	0.95	ND	ND	1.10	1.27	ND	19.52	5.62

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
2 Day High	2A10H5	2.49	6.5	M	0.91	47.1	18	0.37	4.33	362	22.80	6.54
2 Day High	2A10H6	2.19	6	M	1.01	40.3	17	0.52	2.68	ND	13.54	8.31
7 Day Control	7A2C1	0.66	4.6	J	0.68	29.6	18	0.95	0.46	ND	28.53	4.74
7 Day Control	7A2C2	2.89	6.4	M	1.10	28.5	18	2.64	1.01	ND	14.77	5.74
7 Day Control	7A2C3	2.26	6	M	1.05	37.2	20	2.09	1.28	ND	12.20	7.69
7 Day Control	7A2C4	0.92	4.6	F	0.95	31.2	21	2.57	0.31	ND	64.16	1.41
7 Day Control	7A2C5	0.71	4.6	F	0.73	35.6	22	3.22	0.39	ND	4.23	2.15
7 Day Control	7A2C6	0.99	4.9	F	0.84	46.1	19	1.77	0.42	ND	64.16	1.41
7 Day Control	7A4C1	0.81	4.5	F	0.89	33.1	23	1.53	0.60	445	2.94	5.45
7 Day Control	7A4C2	0.80	4.5	F	0.88	35.6	25	2.58	0.56	ND	1.28	2.69
7 Day Control	7A4C3	1.17	5	M	0.93	56.5	22	4.05	0.45	351	1.96	5.85
7 Day Control	7A4C4	0.63	4	F	0.98	31.7	21	0.85	1.01	409	13.75	6.92
7 Day Control	7A4C5	0.97	4.8	F	0.88	33.0	22	3.39	0.86	368	1.28	2.69
7 Day Control	7A4C6	0.60	4.1	J	0.88	39.6	21	1.42	0.65	ND	1.44	6.45
7 Day Control	7A8C1	1.06	4.3	F	1.33	53.4	20	0.59	1.02	409	13.51	6.40
7 Day Control	7A8C2	0.66	4.5	F	0.73	39.2	17	1.90	0.60	ND	13.51	6.40
7 Day Control	7A8C3	1.02	5	F	0.82	33.0	24	2.43	0.26	ND	13.51	6.40
7 Day Control	7A8C4	2.44	6.3	M	0.98	47.3	21	0.86	2.15	595	0.72	6.71
7 Day Control	7A8C5	0.92	4.9	J	0.78	36.5	21	0.62	0.82	620	1.18	4.61
7 Day Control	7A8C6	0.85	4.6	F	0.88	35.7	25	1.43	0.51	646	13.51	6.40
7 Day Control	7A12C1	1.16	5.1	F	0.87	29.3	19	1.53	0.95	484	45.23	3.15
7 Day Control	7A12C2	1.00	4.8	F	0.91	26.3	11	1.24	1.07	566	45.23	3.15
7 Day Control	7A12C3	0.84	4.7	F	0.81	20.4	15	3.45	0.36	432	1.87	4.68
7 Day Control	7A12C4	0.69	4.4	F	0.81	31.8	20	2.54	0.84	ND	45.23	3.15
7 Day Control	7A12C5	2.58	6.1	M	1.14	54.4	19	0.97	1.90	ND	30.93	5.07

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
7 Day Control	7A12C6	0.78	4.4	F	0.92	39.2	25	1.92	0.78	ND	45.23	3.15
7 Day Low	7A1L1	0.87	4.9	M	0.74	ND	ND	0.95	1.74	ND	1.01	5.64
7 Day Low	7A1L2	2.17	5.9	F	1.06	55.6	20	2.99	0.85	ND	120.85	1.66
7 Day Low	7A1L3	1.86	5.7	F	1.01	50.2	22	2.19	1.26	362	12.57	5.71
7 Day Low	7A1L4	0.65	4	F	1.02	ND	ND	1.44	1.44	ND	0.97	4.95
7 Day Low	7A1L5	0.87	4.8	F	0.78	ND	ND	0.56	0.58	ND	2.98	2.37
7 Day Low	7A1L6	0.62	4.3	F	0.78	ND	ND	0.53	0.15	ND	120.85	1.66
7 Day Low	7A6L1	1.25	5.2	F	0.89	ND	ND	0.60	0.35	ND	35.34	6.29
7 Day Low	7A6L2	2.49	6.3	M	0.99	ND	ND	3.31	1.18	ND	115.13	1.71
7 Day Low	7A6L3	1.26	5.1	F	0.95	29.2	30	1.09	0.53	469	25.66	8.83
7 Day Low	7A6L4	1.62	5.5	F	0.97	ND	ND	1.62	0.74	ND	35.49	5.72
7 Day Low	7A6L5	1.59	5.5	F	0.96	39.8	34	0.58	0.43	615	0.43	7.74
7 Day Low	7A6L6	1.78	5.7	F	0.96	ND	ND	1.02	1.02	ND	46.95	5.62
7 Day Low	7A7L1	1.63	5	F	1.30	ND	ND	0.31	0.49	733	1.38	7.03
7 Day Low	7A7L2	3.18	6.4	M	1.21	52.2	21	2.88	1.67	ND	12.51	8.48
7 Day Low	7A7L3	1.78	5.5	M	1.07	ND	ND	6.18	0.74	ND	1.38	7.03
7 Day Low	7A7L4	2.15	5.7	M	1.16	ND	ND	0.51	1.53	ND	42.63	7.92
7 Day Low	7A7L5	0.81	4.1	F	1.17	39.8	28	2.42	0.53	626	1.15	5.07
7 Day Low	7A7L6	1.00	4.7	F	0.97	ND	ND	2.34	0.99	ND	24.60	6.23
7 Day Low	7A11L1	1.20	5	F	0.96	ND	ND	1.18	0.90	ND	43.84	7.79
7 Day Low	7A11L2	0.67	4	J	1.05	ND	ND	3.58	0.47	ND	1.01	5.77
7 Day Low	7A11L3	0.69	4.3	J	0.86	ND	ND	1.30	0.69	ND	22.07	8.22
7 Day Low	7A11L4	0.90	4.5	F	0.99	35.7	28	0.81	0.82	639	1.01	5.77
7 Day Low	7A11L5	0.81	4.6	F	0.83	ND	ND	4.33	0.28	ND	22.07	8.22
7 Day Low	7A11L6	0.50	3.7	J	0.98	43.3	20	5.80	0.11	420	43.84	7.79

Treatment	Sample ID	Weight (g)	Total fork length (cm)	Sex	FCF	PAGE (%)	Mucous cell counts	Na+/K+-ATPase Activity ($\Delta\mu mol.hr.^{-1}mg\ protein^{-1}$)	Gill protein ($mg.ml^{-1}$)	Hepatocyte count (per 0.16 mm^2 FOV)	TBARS (nmol.mg $protein^{-1}$)	Liver Protein ($mg.ml^{-1}$)
7 Day High	7A3H1	1.26	5.4	F	0.80	ND	ND	2.93	0.66	ND	29.13	7.73
7 Day High	7A3H2	1.96	5.7	F	1.06	ND	29	2.49	1.18	390	44.13	8.74
7 Day High	7A3H3	0.62	4.4	F	0.73	ND	ND	1.37	0.75	ND	1.06	5.57
7 Day High	7A3H4	1.21	5	F	0.97	49.1	30	0.99	0.60	ND	6.35	4.96
7 Day High	7A3H5	0.98	4.6	F	1.00	39.2	ND	1.30	1.02	ND	2.89	5.35
7 Day High	7A3H6	0.89	4.5	F	0.98	ND	ND	4.87	0.36	ND	70.18	4.38
7 Day High	7A5H1	1.63	5.7	F	0.88	ND	ND	1.50	0.99	ND	0.91	6.01
7 Day High	7A5H2	1.11	4.8	F	1.00	ND	ND	2.43	0.61	ND	0.91	6.01
7 Day High	7A5H3	1.46	5.4	F	0.92	ND	ND	0.69	0.89	ND	3.98	6.84
7 Day High	7A5H4	1.99	5.9	M	0.97	50.1	29	2.75	1.24	ND	25.56	4.82
7 Day High	7A5H5	0.91	4.5	F	1.00	38.9	24	1.25	0.64	ND	1.35	6.09
7 Day High	7A5H6	0.54	3.8	J	0.98	ND	ND	1.52	0.55	ND	1.35	6.09
7 Day High	7A9H1	1.69	5.3	F	1.13	ND	ND	1.61	1.24	ND	13.85	10.02
7 Day High	7A9H2	1.85	5.5	M	1.11	ND	ND	1.79	1.26	ND	22.83	4.83
7 Day High	7A9H3	0.83	4.8	F	0.75	ND	ND	2.57	0.52	ND	4.39	5.53
7 Day High	7A9H4	0.83	4.7	J	0.80	ND	ND	1.04	0.86	ND	1.70	6.92
7 Day High	7A9H5	2.56	6.4	M	0.97	45.7	41	2.64	1.84	633	9.31	4.95
7 Day High	7A9H6	0.80	4.6	F	0.82	50.8	20	2.58	0.63	425	3.24	3.49
7 Day High	7A10H1	1.93	5.6	F	1.10	40.2	25	1.04	1.52	487	2.43	5.13
7 Day High	7A10H2	1.91	5.8	M	0.98	ND	ND	2.36	1.65	ND	2.13	6.78
7 Day High	7A10H3	1.04	5	F	0.83	ND	ND	2.65	0.29	ND	2.43	5.13
7 Day High	7A10H4	0.85	4.7	F	0.82	31.1	38	1.85	1.02	ND	33.89	6.61
7 Day High	7A10H5	0.56	4.1	F	0.81	ND	ND	2.12	0.41	ND	19.35	5.00
7 Day High	7A10H6	0.54	3.7	F	1.08	ND	ND	3.86	0.26	ND	2.13	6.78

Appendix G: Abbreviations

AChe	Acetyl cholinesterase activity
a.e.	Acid equivalent. Glphosate active ingredient derivede from parent material → IPA salt
ANEO	Alkylamine ethoxylates
ANOVA	Analysis of Variance
ASE	Accelerated solvent extraction
BET	Basal epithelial thickness
BSA	Bovine serum albumin
Chl α	Chlorophyll α
CST	Central standard time
DF	Dilution factor
DFO	Department of Fisheries and Oceans
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DT50	Amount of time required for 50% of the initial concentration to dissipate.
EC50	Effects concentration that causes adverse effects in 50% of the population
EEC	Environmental exposure concentration
ELA	Experimental Lakes Area
FCF	Fulton's condition factor
FWI	Freshwater Institute
GEE	Generalized estimating equation
H&E	Hematoxylin and Eosin
HPLC	High performance liquid chromatography
HQ	Hazard quotient
IPA salt	Isopropaline (IPA) salt
LC/MS/MS	Liquid chromatography with tandem mass spectrometric detection methods
LEACA	Laboratory of Expertise for Aquatic Chemical Analysis
LC50	Lethal concentration required to kill 50% of the population
LOQ	Limit of quantification
MeOH	Methanol
PAGE	Portion of gills available for gas exchange
PAS	Periodic Acid - Schiff

PMRA	Pest Management Regulatory Agency
POEA	Polyethoxylated tallow amine
Redox	Oxidation-reduction potential
SEID	Homogenization buffer: sucrose, EDTA, imidazole, deoxycholic acid
SLL	Secondary lamellae length
SSD	Species sensitivity distribution curve
Susp C	Suspended carbon
Susp N	Suspended nitrogen
Susp P	Suspended phosphorus
TBARS	Thiobarbituric reactive substances
TDN	Total dissolved nitrogen
TDP	Total dissolved phosphorus
TOC	Total organic carbon
TOX	Toxicity
TSS	Total suspended solids
USEPA	United States Environmental